Identification and therapeutic application of molecular parallels between parasites, parasitic vectors and snake venom

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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January 2014

Abstract

Neglected tropical diseases (NTDs) are a group of conditions that exert disability and poverty on populations that comprise the world's poorest billion people. These conditions, although caused by different organisms and cause distinct disease, they share geographical distribution within tropical regions, occur during similar ecological conditions and most importantly have similar biological mechanisms that are utilized to facilitate the pathology of these diseases. Proteolytic enzymes like proteases are used in many biological mechanisms such as, migration through tissue and cellular compartments; haemoglobin digestion, evasion of immune system responses and cause necrosis and fibrosis to vital tissues and organs. Genomic, transcriptomic, and proteomic studies on parasites (*S. mansoni* and *F. hepatica),* parasitic vector (*An. gambiae)* salivary glands and snake venom show that these diverse pathogens appear to be utilizing similar molecules to perform similar biological mechanisms. Therefore, it is of interest to ascertain whether a cross-cutting approach in research could facilitate a better understanding of these diseases. Therefore, the initial aim of this work was to investigate molecular parallels of the mechanisms used by these tropical disease pathogens, including parasites, snake venom toxins, and haematophagic parasite vectors, to access their host's blood stream. Using a bioinformatics-led approach, in combination with immunological and proteomic analyses, this study demonstrated the presence of similar compounds between shared molecular molecules (serine proteases and other proteins) causing pathology in parasites, parasitic vectors and snake venom. This similarity was not only at the bioinformatics level, but presence of cross-reactivity toward parasite proteins was detected using antivenoms and toxin-specific antibodies. In addition, sera collected from patients infected with *S.mansoni* exhibited an immune response to snake venom. One of this study aims was to investigate possibilities of using homologous proteins in parasitic vectors and snake venom as therapeutic applications. For this purpose, chimeric epitopes of homologous snake venom (*Echis ocellatus*) and mosquito salivary proteins were designed as primary vaccine that could be boosted by mosquito bites. If succeeded, this method would prevent, or at least reduce, the devastating pathology and death caused by snake venom at a low cost, with limited logistical complications.

ACKNOWLEDGEMENTS

My grateful thanks go to my supervisor Dr. Rob Harrison for all of his invaluable help and guidance during my PhD. His assistance and encouragement have been integral for my progression.

I would also like to thank my co-supervisor Dr. Simon Wagstaff for his support and guidance in bioinformatics.

General thanks go to Paul Rowley for his expert herpetological assistance and Dr. Gavin Laing for conducting the LC-MS/MS analysis.

Big thanks go to Nick, Rachel, Camila, Gareth and Fiona for being great office mates.

Special thanks go to my family. Words cannot express how grateful I am to my mother, father and Alya my little sister for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far. I would also like to thank all of my family and friends who supported me, and incentivized me to strive towards my goal. At the end I would like express a special appreciation to my beloved husband Raeed who sacrifice his career and live caming all the way with me to this point and was always my support in the moments when there was no one to answer my queries. And not to forget my sweethearts, (my children) Saja and Abdulrahman, for being patient with me during my stressful days. I would like to apologize to them for the times I have been away from them when they needed me.

CONTENTS

ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
APS	Ammonium persulphate
BLAST	Basic Local Alignment Search Tool
° C	Degrees centigrade
CTL	C-type lectin
CLP	CTL-like protein
DAB	3,3'-diaminobenzidine
dH_2O	Distilled water
DTT	Dithiothreitol
EST	Expressed sequence tag
HPLC	High performance liquid chromatography
IAN	Iodoacetamide
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IVIG	Intravenous immunoglobulin
kDa	Kilo Dalton
LAO	L-amino acid oxidase
mM	Millimolar concentration
MS	Mass spectrometry
Min	Minutes
PBS	Phosphate buffer saline

PLA_2	Phospholipase A_2
PLOB	Protein loading buffer
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SP	Serine protease
SVMP	Snake venom metalloprotease
TBST	Tris-buffered saline-Tween 20
TEMED	Tetramethylethylenediamine
v	Voltage

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Chapter 1 General introduction

1.1 Introduction

Neglected tropical diseases (NTDs) are a group of disfiguring and disabling conditions that are among the most widespread infections affecting the world's most impoverished regions (Daumerie et al. 2010). These diseases are caused by variety of organisms such as viruses, bacteria, parasitic protozoa and worms, and other conditions such as Buruli ulcer and snakebites (Herrera et al., 2011; Kasturiratne et al., 2008; WHO 2012). Despite the fact that NTDs are clinically diverse, they are grouped as poverty-related diseases since they flourish in settings of extreme poverty in tropical regions, where they tend to coexist. NTDs are ancient diseases that have plagued humanity for centuries; they now primarily affect low-income countries throughout sub-Saharan Africa, Asia, and Latin America. The fact that they affect low-income countries, mainly the poorest populations, living in remote, rural areas, urban slums or conflict zones, lower the global attention to these diseases and thus make them neglected.

The majority of these diseases are transmitted through insect (mosquitoes, black flies, sand flies, and tsetse flies) and snail vectors. While others, spread via contaminated water or worm infested soil. While NTDs can be fatal, their primary importance is that they typically result in chronic disabilities, disfigurements, complications in pregnancy, impaired physical and cognitive development, that eventually limit adult productivity in the workforce (Herrera et al., 2011). In fact, NTDs rank closely with diarrheal diseases and malaria in the number of years lost to disability and premature death, or disability adjusted life-years (DALYs) (Hotez et al., 2007). Thus, NTDs enhance poverty by placing huge burdens on both the health and economic development of low-income countries.

Treatments for some NTDs are available, however, many have significant adverse side effects and are not well tolerated by patients (Daumerie et al. 2010). Given the limited access to existing treatments, rapid reinfection following treatment, and drug resistance (Daumerie et al. 2010; Health & Diseases 2007; Hotez et al. 2007) NTDs, and the billions of people affected by them, are worthy of

increased attention regarding the need for novel interventions to eliminate and treat these diseases.

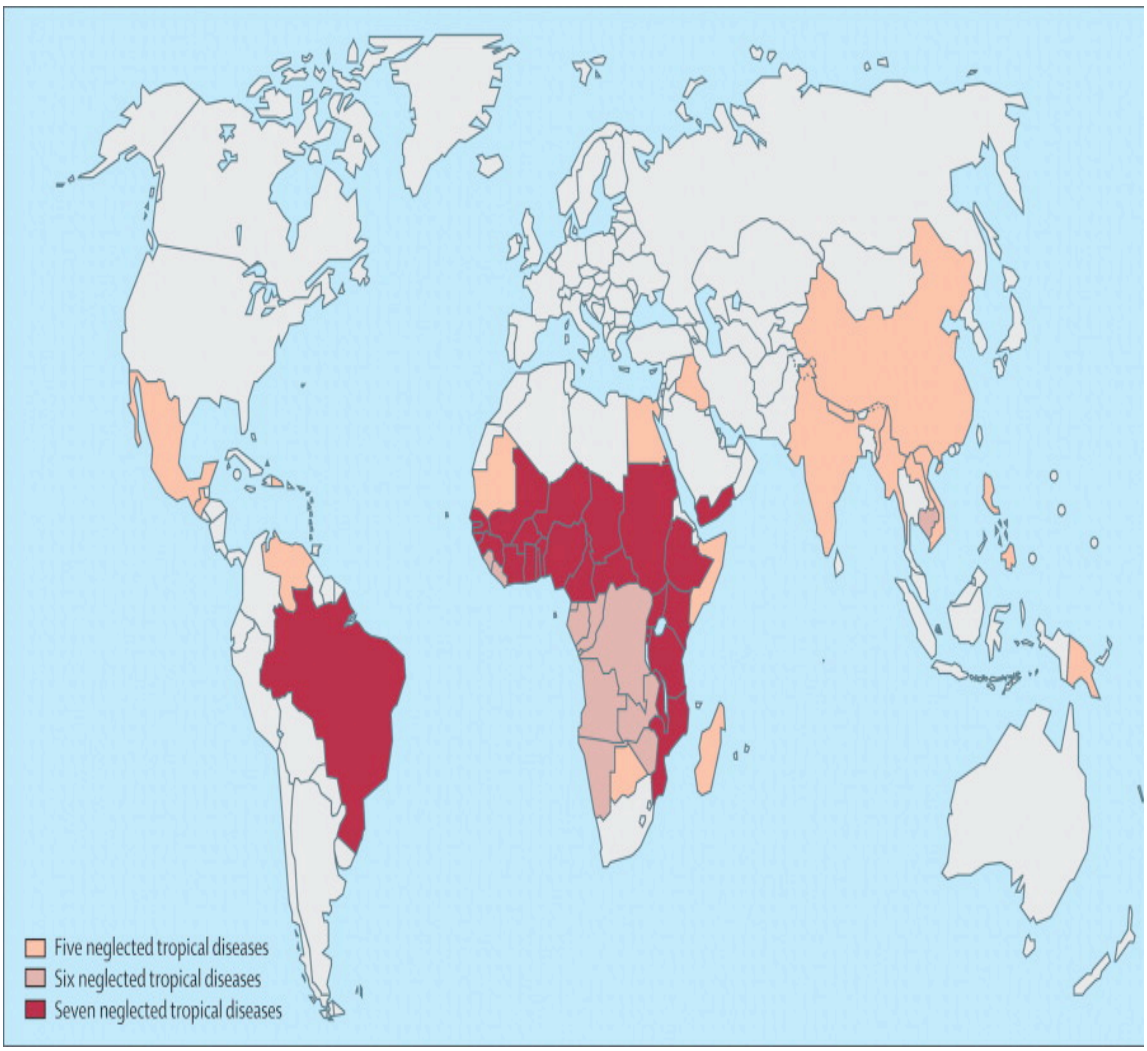


Figure 0.1 NTDs distribution: Map showing geographic overlap and distribution of the seven most common neglected tropical diseases (the soil-transmitted helminth infections (hookworm, ascariasis, and trichuriasis); lymphatic filariasis; schistosomiasis; and diseases that cause blindness—trachoma and onchocerciasis (river blindness) (figure from; Hotez et al. 2009).

1.2 Geographical

1.2.1 Epidemiological burden of NTDs

Until the last decade, NTDs were low on national and international health agendas as priority was given to the "big three": HIV/AIDS, malaria, and tuberculosis (TB) (Molyneux et al., 2005). NTDs are considered as one of the most potent reinforcements of the poverty trap and the world's poorest billion people have at least one of these diseases because NTDs tend to coexist in 56 of 58 endemic countries as illustrated in figure 1.1 (Hotez et al., 2009). Multiple NTDs frequently coexist not just in the same populations, but also within the same individuals (Solomon et al., 2012). Poverty and NTDs fed upon another; as access to disease control intervention and therapeutic services are typically low in the communities vulnerable to NTDs. As described in the following sections, this large population of the world's population becomes anchored into poverty by NTDs.

Trachoma and onchocerciasis, which cause blindness, are prevalent worldwide in about 84 million and 37 million people, respectively (Fenwick 2012, Hotez et al., 2009). A further 120 million people have lymphatic filariasis, and 180,000 cases of leprosy hamper productivity and eliminate normal social life opportunities as a result of their deformability (Fenwick, 2012; WHO 2012). Other NTDs can lead to permanent disfigurement or disability, especially when limbs have to be amputated as a life-saving procedure; for example, over 400,000 amputations are performed annually as a result of snakebite envenoming (Williams et al. 2010). Further examples include 5,000–6,000 cases of buruli ulcer reported annually, out of which 33% of reported cases in Africa reach category III (patient has single > 15 cm in diameter, multiple lesions or lesion(s) at a critical site (eye, breast, genitalia) and osteomyelitis) (WHO, 2012). Human African trypanosomiasis (sleeping sickness) affects 42,000 individuals who are at risk of being severely debilitated and mortality reaches 100% in untreated cases (Simarro et al., 2010). Without post-exposure prophylaxis, rabies causes acute encephalitis and is always fatal, with an estimated 24,000 deaths occuring annually in Africa (Both et al., 2012). Leishmaniasis has a global incidence of 1–1.5 million cases of cutaneous leishmaniasis and 500,000 of visceral leishmaniasis (Bhargava and Sing, 2012), leaving deep and permanent scars or even destroying the mucous membranes of the nose, mouth, and throat. In its

visceral form, it infects vital organs and is fatal without treatment (Bhargava and Sing, 2012). In the Americas 8 million people are infected with Chagas disease (Bern et al., 2011); acute disease can cause young adults to develop myocarditis, pericardial effusion, and/or meningoencephalitis, leaving them out of the labour force (Bern et al., 2011). Among 207 million people with schistosomiasis, children and adolescents tend to have the highest disease intensity (Steinmann et al., 2006); it disrupts their school attendance, causes anaemia and malnutrition, and impairs cognitive development (Hotez et al., 2009). Although nearly eradicated, Guinea-worm disease causes agonizing, severe pain, which can last for extended periods coinciding with the peak agricultural season (Rinaldi, 2009). More than 2.5 billion people – over 40% of the world's population – are now at risk from Dengue as it has emerged as a rapidly spreading vector-borne disease affecting mostly poor, urban populations (Racloz et al., 2012). Over 90% of the global burden of these NTDs is believed to occur in Africa (Molyneux et al., 2005).

1.2.2 NTDs in sub-Saharan Africa

In sub-Saharan Africa (SSA), NTDs are the most common health issues affecting the 500 million impoverished people (Hotez and Kamath, 2009) (Table 2). The collective burden of these diseases may be up to one-half of SSA's malaria disease burden and more than twice that of TB (Hotez and Kamath, 2009).

Almost 50% of SSA's poorest people have hookworm infection (Hotez and Kamath, 2009); of them, 40–50 million school aged children may have impaired physical and intellectual development, and reduce their school performance and attendance as a consequence of the infection. In addition, 7 million pregnant women are threatened by anaemia and, therefore, low foetal birth weight, impaired milk production, and increased risk of death for both mother and infant (Hotez and Kamath, 2009). Schistosomiasis has become the second most prevalent parasitic infection in SSA; around 192 million infected individuals account for 93% of the total global burden of schistosomiasis. Other NTDs such as lymphatic filariasis and onchocerciasis, affect around 46–51 million and 37 million people, respectively (Hotez and Kamath, 2009). Moreover, human African trypanosomiasis and visceral leishmaniasis occur at a prevalence of around 10,000 cases annually. Snakebite

envenoming kills around 7,500-32,000 individuals annually in SSA alone with a devastating impact on the population (Kasturiratne et al. 2008; Harrison et al., 2011). Each one of these NTDs exert a significant disability and reduction on SSA's economy, especially in agricultural productivity that coincide with rainfall and monsoon seasons, as most of the rural poor population depends on agriculture as a source of income and food (Hotez and Kamath, 2009).

Disease	Estimated Population Infected in SSA	Estimated % of SSA Population Infected	Estimated % Global Disease Burden in SSA
Hookworm	198 million	29%[a]	34%[b]
Schistosomiasis	192 million	25%	93%
Ascariasis	173 million	25%a	21%[2b]
Trichuriasis	162 million	24%[a]	27%[b]
Lymphatic filariasis	46–51 million	6%–9%	37%–44%[c]
Onchocerciasis	37 million	5%	>99%
Active trachoma	30 million	3%	48%
Loiasis	≤13 million	1%–2%	100%
Yellow fever	180,000	0.02%	90%
Human African trypanosomiasis	50,000–70,000 (17,000 new cases annually)	<0.01%	100%
Leprosy	30,055 (registered prevalence); 21,037 new cases in 2007	<0.01%	14%
Leishmaniasis (visceral)	19,000–24,000 new cases annually in Sudan and Ethiopia	<0.01	ND
Dracunculiasis	9,585	<0.01%	100%
Buruli ulcer	>4,000	<0.01%	57%

Table 0.1 Ranking of Neglected Tropical Diseases (NTDs) in SSA by Prevalence and Distribution (Hotez & Kamath 2009).

1.3 Ecological

1.3.1 Influence of rainfall on some tropical diseases

NTDs have major impacts on the economic status of the poorest sector of the population worldwide, who depend mainly on agricultural activity and grazing livestock. Therefore, the rainy and monsoon season coincides with an increase in these activities. This coincidence is exemplified by the increase in hospital admissions due to snakebites (Kasturiratne et al., 2008; Molesworth et al., 2003; Mohapatra et al., 2011). In West Africa, the probability of snakebite incidence is increased in the period between March and July at the beginning of the rainy season

and during the harvest period after the peak rains; both periods have intense agricultural activity (figure 2) (Molesworth et al., 2003). Snake irritability, concomitant with the breeding season, is also coincident with the start of the rainy season (Warrell and Arnett, 1976). This is coherent with the hypothesis that heavier rains force snakes to drier areas thus increasing human-snake contact (Molesworth et al., 2003).

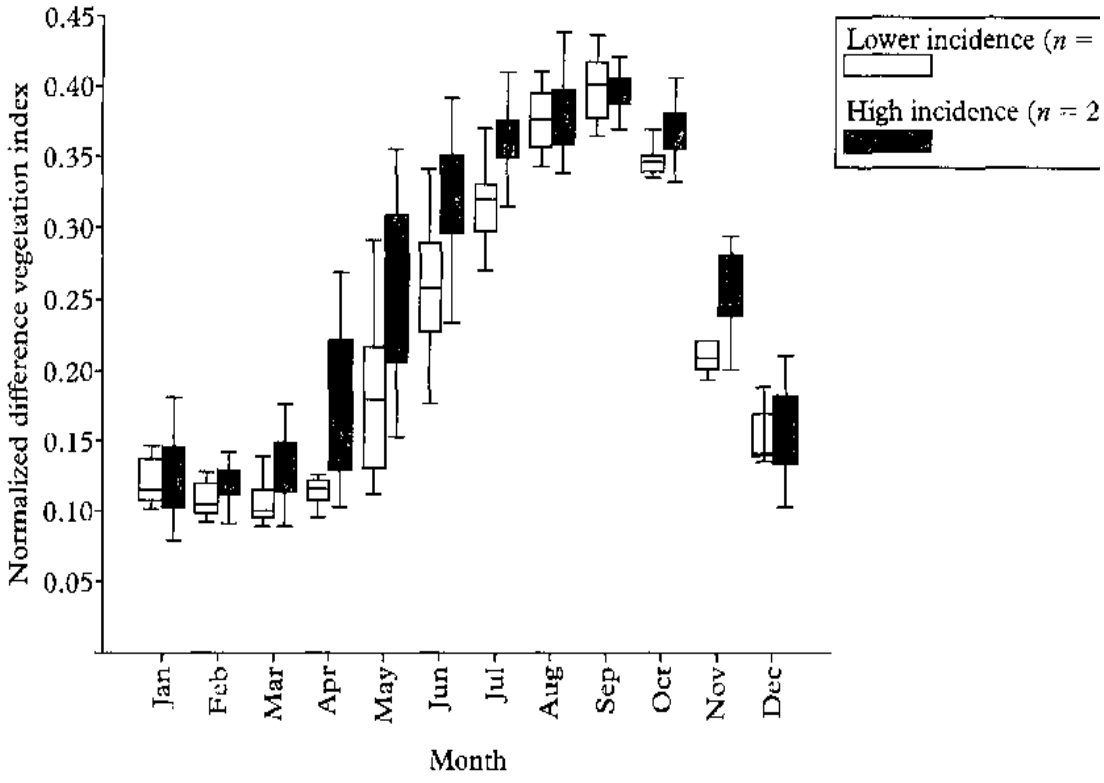


Figure 0.2 Comparison of the distribution of monthly-normalized difference vegetation index (NDVI) and snakebite incidence (with permission from; Molesworth et al. 2003)

Different species of mosquitoes play critical roles in transmission of many tropical diseases (e.g., malaria and many NTDs such as dengue fever and yellow fever). The literature demonstrates a relation between rainfall seasons and mosquito burden. Rainfall facilitates the growth of mosquito populations (Chaves et al., 2012; Chaves et al., 2011). On one hand, continuous rainfall can inhibit mosquito activity and therefore have negative effects on oviposition. On the other, it can lead to increased mosquito abundance via a phase locking i.e. rainfall facilitates the growth of mosquito populations (figure 3) (Chaves and Kitron, 2011).

Considering the above information, one might conclude that there can be an overlapping period where both snakebite incidence and number of mosquitoes increase during the rainy season.

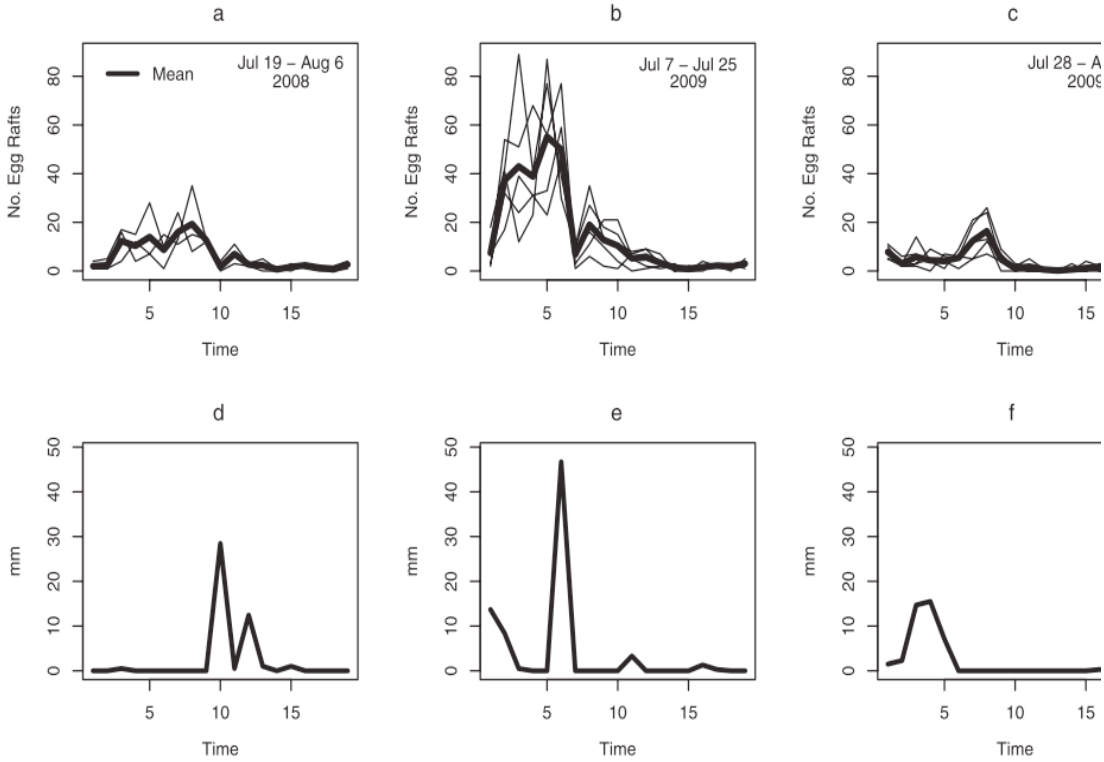


Figure 0.3 Associations between rainfall and mosquito oviposition: Time series (a), (b) and (c) show the average number of egg rafts (thick lines) and individual replicates (thin lines) for observation periods A, B and C, respectively. (d), (e) and (f) show the daily rainfall during the observation periods A, B and C, respectively (with permission from; Chaves & Kitron 2011).

1.4 Biological

1.4.1 NTDs, is there a shared focus?

Most NTD-causing pathogens (parasite, bacteria, virus, or even venom) share a common biological challenge; they must all be able to invade their host. Once on or inside their host, they must rapidly find ingress to their final habitat or pathogenic site – a specific cell type, organ, or vessel. In parasite NTDs this involves migration through host tissue, extracellular matrix, basement membranes, and/or blood or lymph vessel walls and the pathogen must overcome host defence mechanisms, i.e.,

physiochemical, immunological, and behavioural barriers. Given that NTDs share the same geographical distribution and cause high morbidity and mortality on the suffering population, a cross-cutting approach in research may facilitate a better understanding of the co-morbidity of the disperate NTD pathogens.

The next section of this introduction explore similarities between schistosomes, fasciola and snake venom proteins in the context of accessing the intravascular and tissue compartments.

1.4.2 Schistosomiasis

Schistosomes are blood-dwelling parasites that cause schistosomiasis or bilharzia. The main species causing the disease in humans are *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*. Schistosomiasis is, after malaria, the most prevalent serious parasitic disease. Recent epidemiological studies indicate that 200 million people are infected in underdeveloped and developing regions; further, the disease is a growing concern for travellers worldwide (Figure 4.1) (Gryseels et al. 2006). The latest WHO report states that the annual mortality rate due to schistosomiasis could be as high as 200,000 and estimates that 600 million people live in high-risk areas. The loss in DALYs caused by the disease is about 1.53 million (Gryseels et al. 2006).

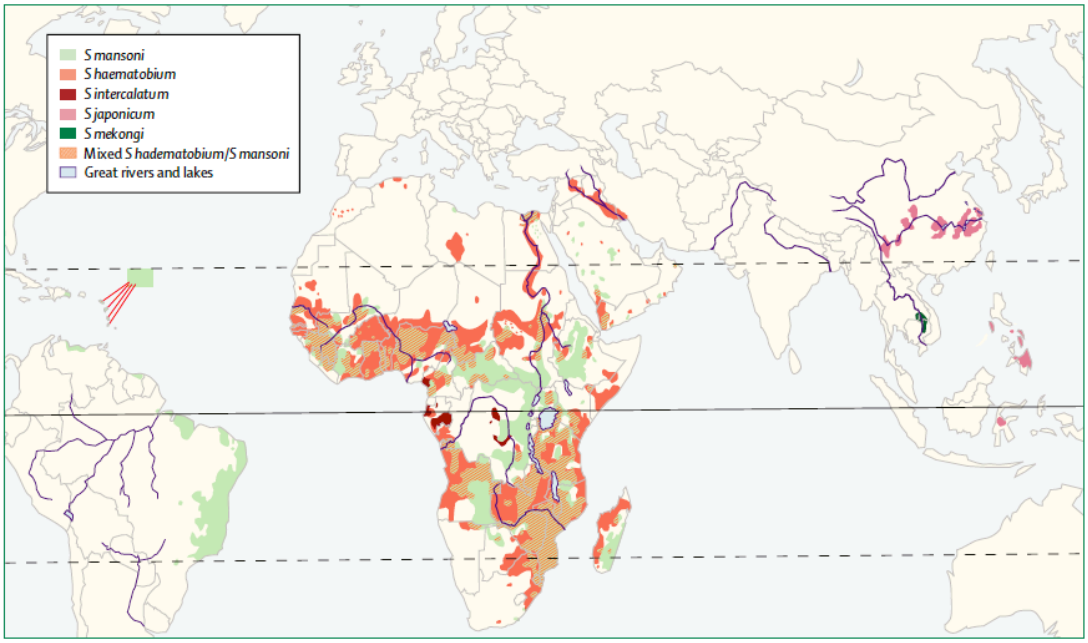


Figure 0.4 Global distribution of schistosomiasis (Gryseels et al. 2006).

1.4.2.1 Schistosome life cycle

Schistosomes have a multi-stage life cycle (Figure 5). Adult worms are 7–20 mm white or greyish in colour. They have a cylindrical body with two terminal suckers (oral and ventral), a complex structured tegument, a digestive tube, and a reproductive system. Schistosomes are distinctive from other trematodes; they are not hermaphrodites as they have separate sexes. The long thin female resides in a groove in the male body called the gynaecophoric channel. After mating, the permanently embraced couple migrates to either the perivesical (*S. haematobium*) or the mesenteric (other species) venous plexus. Schistosomes are blood-dwelling parasites so their residence in blood vessels guarantees a rich source of blood and globulins that they digest through anaerobic glycolysis.

The adult lifespan averages three to five years; however, some may live as long as 30 years. During this time the female may produce as many as 600 billion eggs. The eggs bear ciliated miracidium larvae, which help the egg during migration to the lumen of the bladder (*S. haematobium*) or the intestine (other species) through its proteolytic enzyme secretion. Once in the lumen, the eggs can be excreted by urine or faeces to the outer environment. The embryonated eggs are viable for up to seven days. When the eggs are released into fresh water, the miracidium swims from the hatched egg searching for an intermediate host (fresh water snail). Guided by

light and chemical stimuli, the free-swimming miracidium penetrates the snail and multiplies asexually into multicellular sporocysts. Germ cells within sporocysts continue multiplying and growing, producing thousands of the bifurcated tail cercariae. After four to six weeks of infection the young cercariae emerge from the snail and swim around in water for up to 72 hours seeking for a definitive host. Stimulated by water turbulence, shadow, and chemicals released from human skin, the cercariae attach to the skin of its definitive host in order to initiate infection.

After attachment to human skin, cercariae secrete proteolytic enzymes that breakdown the skin's proteins to facilitate penetration of the cercarial head through the skin layers. As cercariae penetrate the skin they lose the bifurcated tail and transform into migrating schistosomula. The juvenile schistosomulum may remain within the skin for about two days until it locates a post-capillary venule. Upon reaching the capillaries, schistosomula migrate to the lungs where they further develop in order to continue migration to liver sinusoids, approximately within eight to ten days of penetration. After arrival at the liver, the parasite develops an oral sucker through which it feeds on red blood cells. Parasites reach maturity in six to eight weeks from the origin of infection, after which the mature male and female worms pair, the female resides in the gynaecophoric channel to produce eggs, and the life cycle starts again.

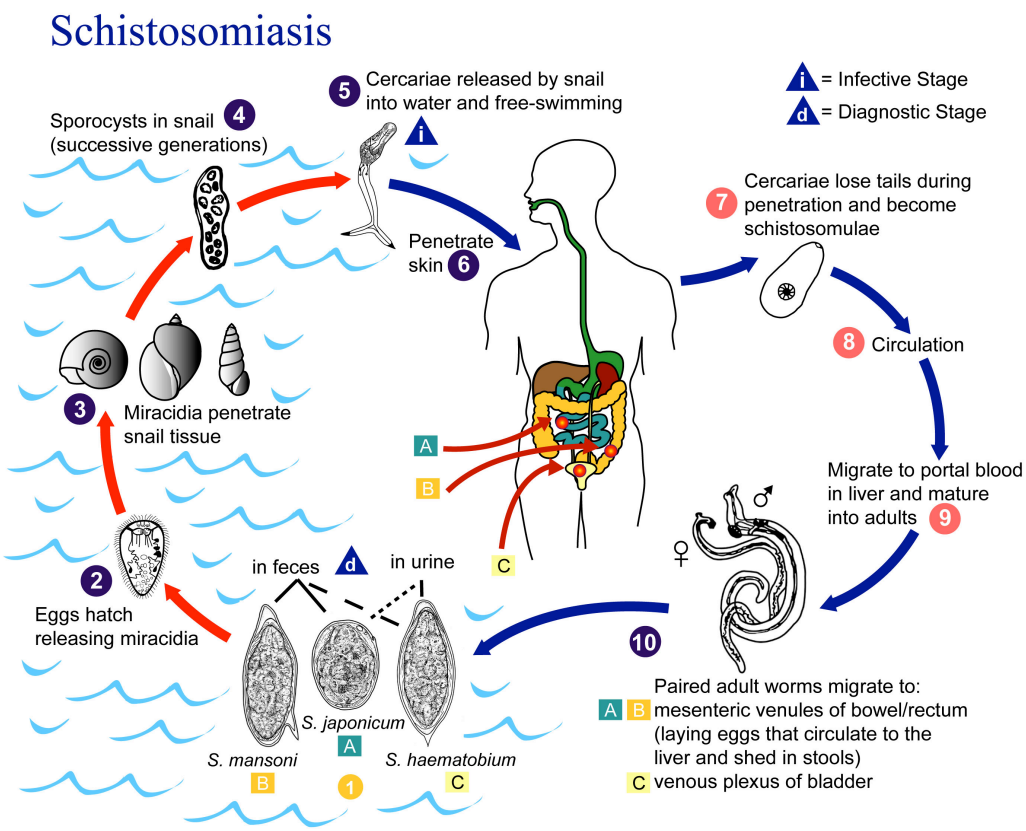


Figure 0.5 Schistosome life cycle. (figure from; http://www.cdc.gov/parasites/schistosomiasis/biology.html)

1.4.2.2 Pathology

1.4.2.2.1 Acute pathology

Skin penetration by schistosome cercariae can provoke a local urticarial rash that may last for a few days. That occurs particularly after primary infection in non-indigenous who visited or migrated to endemic areas as it often goes unrecognized in endemic populations (Gryseels et al., 2006).

Acute schistosomiasis, otherwise known as Katayama fever, is a systemic hypersensitivity, serum sickness-like illness, which develops against released antigens during migration of the schistosomula and early oviposition (Bottieau et al., 2006). Clinical onsets occur after several weeks of initial infection in some, but not most, newly infected patients. The disease is associated with marked peripheral eosinophilia, fever, fatigue, myalgia, malaise, non-productive cough,

hepatosplenomegaly, and patchy infiltrates on chest radiography (Gryseels et al., 2006). Later, migration and positioning of the mature worm provoke abdominal symptoms. Although the syndrome can be fatal, most patients show spontaneous recovery over several weeks. Katayama fever is most common with *S. mansoni* and *S. japonicum*, and is most likely to appear in primary infected individuals with heavy infection (Gryseels et al., 2006). It is common in tourists, travellers, and people accidentally exposed to transmission foci (Bottieau et al., 2006).

1.4.2.2.2 Chronic pathology

Chronic schistosomiasis occurs through pathogenic reaction to eggs trapped in the patient's tissue rather than to adult worms. Disease manifestation appears a number of years after initial infection. The pathogenic reaction is characterized as a cellular, granulomatous inflammation around trapped eggs in the intestine, liver (*S. mansoni* and *S. japonicum*) or urinary bladder (*S. haematobium*), which is progressively replaced by fibrotic deposits. Eggs are trapped in tissues during perivesical or peri-intestinal migration, or after embolization in the liver, spleen, lungs, or cerebrospinal system. Proteolytic enzymes secreted by the eggs during this process provoke an eosinophilic inflammatory reaction that establishes the granuloma. The severity of symptoms varies according to the intensity of the infection and the immune status of the infected patient.

1.4.2.3 Schistosomiasis diagnosis (table 2)

Schistosomiasis can be diagnosed through direct or indirect methods. Direct methods depend on the detection of parasite eggs in excreta or tissue of infected individuals or detection of parasite-derived material in the patient's circulation or excreta. Indirect methods rely on the detection of schistosome-specific antibodies in patient blood samples (Doenhoff et al., 2004).

Category	Method	Detection of	Applicable for infection with		
			S. haem.	*S. mans.*	*S.jap.*
Direct: Parasitological	Examination of urine, stool, rectal mucosa	Eggs	+	+	+
Indirect: Assessment of specific pathology through clinical, biochemical and immunological disease markers	Looking/asking for symptoms and signs	Gross haematouria,	+		
		bloody diarrhoea,		+	**(+)**
		microhaematuria,	+		
		proteinuria,	+	**(+)**	
		leukocyturia,	+		
		occult blood in stool		+	**(+)**
	Looking for organ involvement	Sonographical alteration*	+	+	+
	Assessment of altered immune function	Altered liver haemodynamics and collagen synthesis		+	**(+)**
		Altered cell-mediated immunity	+	+	+
Indirect, Direct: Immunological	Serology	Specific antibodies	+	+	+
	In vivo immediate and delayed-type hypersensitivity	Schistosome-derived antigens	+	+	+
		Previous sensitization	+	+	+

*Of liver and spleen, and lower and upper urinary tract, respectively.

Table 0.2 Categories of diagnostic methods, (+) only inconclusive data exist (table from; Feldmeier et al., 1993).

1.4.2.4 Brief summary of treatment and control

Over the past few decades, schistosomiasis control strategies have shifted fundamentally due to production of schistosomicides like praziquantel (PZQ) (Gryseels et al., 2006). The focus on transmission control through the intermediate host has been altered by controlling morbidity using population-based chemotherapy (Liu et al., 2011). Although this strategy has the advantage of quick results, it needs

careful and long-term planning to ensure sustainability and progression to face the challenging stages of infection and control of transmission (Gryseels et al., 2006).

Three main drugs have been used to treat schistosomiasis; metrifonate (targeting *S. haematobium*), oxamniquine (targeting S. *mansoni*), and PZQ (for all human species) (Liu et al., 2011). Because of its broader spectrum, schistosomiasis is readily treated by a single annual oral dose of PZQ, an acylated quinoline-pyrazine. However, potential resistance of schistosomiasis to PZQ has recently become a concern, thereby increasing the focus on the need for alternatives. As with other major parasitic diseases, vaccine development research is ongoing and extensive. Researchers are trying to develop a schistosomiasis vaccine that will prevent the parasite from completing its life cycle in humans.

1.4.2.5 The saga of schistosome migration

Invasion of skin is the initial event in infection of the vertebrate host by schistosome larvae. Skin is a formidable barrier made by connected cells of the epidermis, the dermal-epidermal basement membrane, and the extracellular matrix of the dermis. Cercariae are stimulated to penetrate the skin by interaction with medium-chain fatty acids on the skin surface (Stirewalt et al., 1965; McKerrow et al., 1985; Haeberlein and Haas, 2008). After host identification, ventral suckers of the cercariae aid their attachment to the epidermis. Schistosome cercariae have a specialized head organ that has the ability to stretch and retract, and is used to find a suitable place for invasion (He et al., 2005). The external opening of the acetabular gland ducts is present on the disc-like apex of the head. The acetabular gland secretions are sticky and released into the microenvironment in large quantities after stimulation (He et al., 2005).

Proteases released from the acetabular glands initially degrade keratin at the skin surface (McKerrow et al., 1985). Once on the skin, cercariae (transforming into schistosomula) may cross the epidermis directly or first migrate down a hair shaft. The active component in acetabular gland secretions, cercarial elastase, is responsible for the subsequent proteolytic activity of invading larvae (McKerrow and

Salter, 2002); it diffuses widely ahead of the invading larvae (Stirewalt and Walters, 1973). Nevertheless, other surface-associated proteases, namely serine proteases and metalloproteases (e.g., SmPepM8), of the schistosomula in coordination with the cercarial enzyme may facilitate subsequent vascular invasion (Silva et al., 2011; McKerrow et al., 1985; Curwen et al., 2006). 3-day-old schistosomulea, which now lack preacetabular-gland contents, are as effective as cercariae at degrading the basement-membrane-like extracellular matrix of endothelial cells (Curwen and Wilson 2003; McKerrow, Jones, et al. 1985).

Finally, whilst migrating to the blood venules, schistosomula have to degrade the basement membrane that is the final barrier to dermal entry. In the basement membrane, the lamina densa contains two forms of collagen IV and collagen VII (McKerrow et al., 1985; Curwen and Wilson, 2003; Ruppel et al., 2004). The major obstacles in the dermis itself are collagen, elastin, and reticulin fibres. Basement-membrane degradation would enable parasite invasion not only of the epidermal basement membrane, but of small dermal vessels as well. During this phase serine proteinases correspond to cleavage of a globular heparin-binding region from the 'arms' of the laminin molecule (McKerrow et al., 1985). Following entry to the dermis, parasite invasion would be facilitated by degradation of elastin (McKerrow et al., 1983, 1985). Furthermore, metalloprotease activity has already been demonstrated in secretions from maturing schistosomula after the first 24 hours (Curwen and Wilson, 2003).

1.4.3 Fascioliasis

Food-borne trematodiasis account for over 665,000 DALYs worldwide; fascioliasis accounting for 35,000 of these (Fürst et al., 2012). Fascioliasis is a zoonotic disease that infects humans through consumption of water plants (e.g., watercress) contaminated by *F. hepatica* or *F. gigantica metacercaria* (Cabada and White, 2012). Globally, fascioliasis is the most widespread food-borne trematode infection (Fürst et al., 2012). Reports have shown significant numbers of disease

cases from Southeast Asia, Northern Africa, the Middle East, and South America (Cabada and White, 2012). Several factors are likely to explain the expanding endemic territories of fascioliasis, namely human migration, poverty and poor living conditions, animal trade, and environmental changes (Cabada and White, 2012).

1.4.3.1 Fasciola life cycle (Figure 6)

Embryonated ova hatch in fresh water, where newly emerged meracidia infect lymnaeid snails, the obligatory intermediate host (Cabada and White, 2012). After a period of development, cercariae emerge from the snails to form the encysted metacercariae in water or on aquatic plants. Mammals, including humans, are infected when they ingest infected aquatic plants or water (Cabada and White, 2012). After ingestion, the newly excysted juveniles actively penetrate and transverse the gut wall into the peritoneal cavity within two or three hours (Cancela et al., 2010). Four or five days post-infection, the parasites reach the liver, penetrate the parenchyma and continue burrowing for several weeks. The parasites mature and start to release eggs within the major bile ducts. Eggs can be found in the bile and faeces eight weeks post-infection (Cancela et al., 2010).

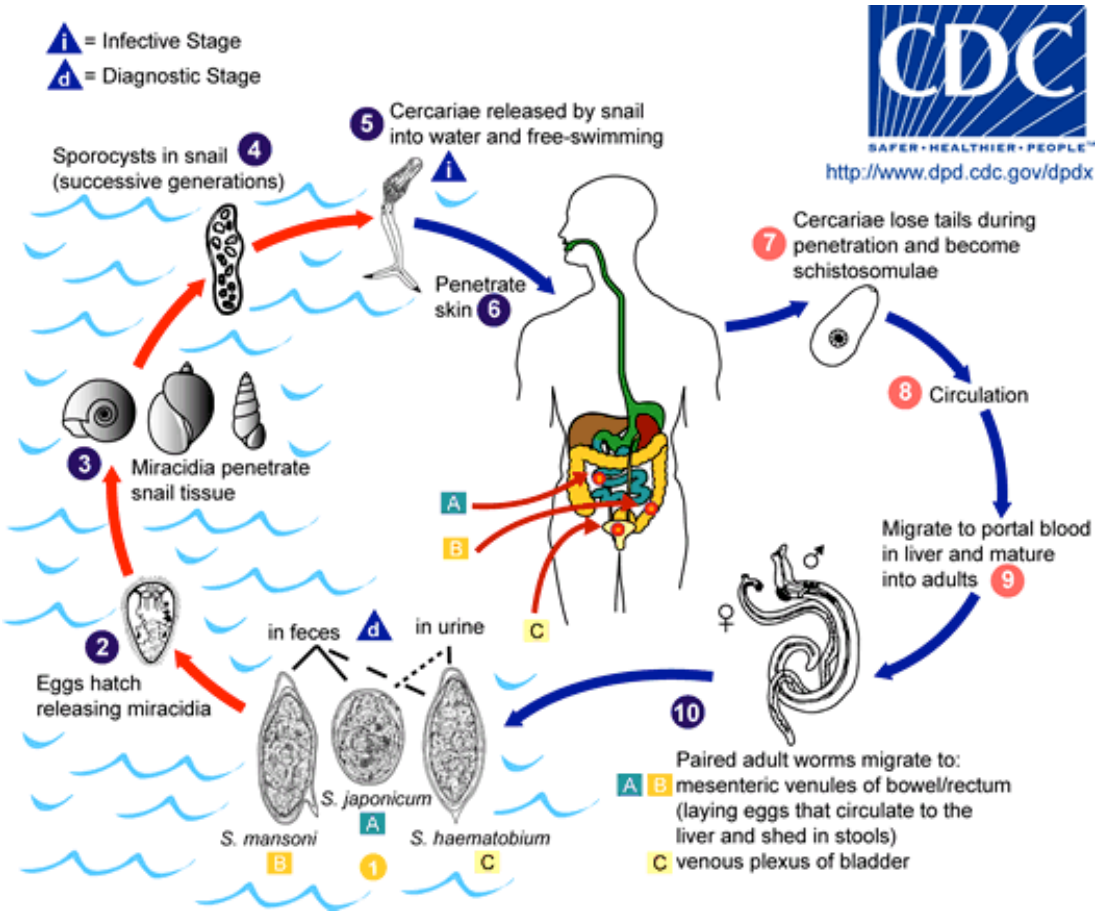


Figure 0.6 Fasciola life cycle. (figure from; http://www.cdc.gov/parasites/fasciola/biology.html)

The primary roles for stage specific proteases and antioxidant enzymes in the early infection have been elucidated (Tkalcevic et al., 1995) who identified proteases in newly excysted juvenile stage of fasciola. Proteomic studies were able to reveal important proteases in different *F. hepatica* stages. Of the recognized proteases, cysteine and asparaginyl proteases were detected (Kasný et al., 2009). Similar to other trematodes, these proteases are believed to be involved in tissue invasion, immune system evasion, and haemoglobin digestion.

1.4.3.2 Pathology

Food-borne trematodes do not have the ability to replicate in the human host. This makes them different from protozoan parasite infection, such as the malaria parasite *Plasmodium* spp., which can replicate within infected individuals (Keiser and Utzinger, 2009). Thus, morbidity due to food-borne trematodiasis and other trematode-borne diseases is related to infection intensity and worm burden (Keiser & Utzinger 2009). In addition, worm burden has a direct effect on inflammatory lesions and damage of tissue and target organs. Common symptoms of fascioliasis includes dyspepsia, fever, right upper quadrant pain, anorexia, hepatomegaly, splenomegaly, ascites, urticaria, respiratory symptoms, and jaundice, which might be seen in the acute stage of fascioliasis (Keiser and Utzinger, 2009). In the chronic stage, when the parasites enter into the biliary tree, symptoms such as biliary colic, intermittent jaundice, epigastric pain, nausea, fatty food intolerance, cholangitis, acute pancreatitis, and cholecystitis may present (Keiser and Utzinger, 2009).

1.4.3.3 Diagnosis

Diagnosis of fascioliasis is performed through three main approaches: direct parasitological diagnosis, immunodiagnosis, and molecular diagnostic tests (Cabada and White, 2012). Direct parasitological diagnosis depends on detection of parasite eggs in stool, sputum, or other biofluids (Fürst et al., 2012). Indirect immunodiagnosis detects parasite antigens or antibodies in patient blood. Complementary tools are available for the diagnosis of the hepatobiliary parasitic infections, for example, ultrasound, computer tomography, magnetic resonance imaging, and tissue harmonic imaging (Keiser and Utzinger, 2009).

1.4.3.4 Brief summary of treatment and control

Public health interventions are playing a major role in the control of fascioliasis. To reduce the prevalence and intensity of infections, and as a result morbidity and mortality, chemotherapy (mainly by triclabendazole), improved access to adequate sanitation, the use of chemical fertilizers and food inspections,

information, education, and communication campaigns have been employed. The consumption of raw or undercooked aquatic products increases the risk of acquiring fascioliasis; therefore, changing this behaviour and raising public awareness to its risks is a key factor in controlling the disease. No vaccines are available yet for the prevention of fascioliasis, however, progressing gene discovery and proteome research may decrease the gap to develop effective vaccines and the next generation trematocidal drugs.

1.4.4 Snake envenoming

Mortality due snakebite is a neglected health issue affecting the rural poor in low-income tropical countries (Harrison et al., 2011). It kills over 50,000 individuals in Asia (Mohapatra et al., 2011) and 7,500 in SSA (Chippaux, 2011) annually; globally, it is estimated that as many as 95,000 people die every year as a result of snakebite envenoming (Kasturiratne et al., 2008) (Figure 7). The catastrophic effects behind snakebites are due to the fact that the majority of the affected population are the least able to access or afford effective treatment (Harrison et al., 2011). Recently, snakebite envenoming has been designated by WHO as a NTD (WHO, 2013).

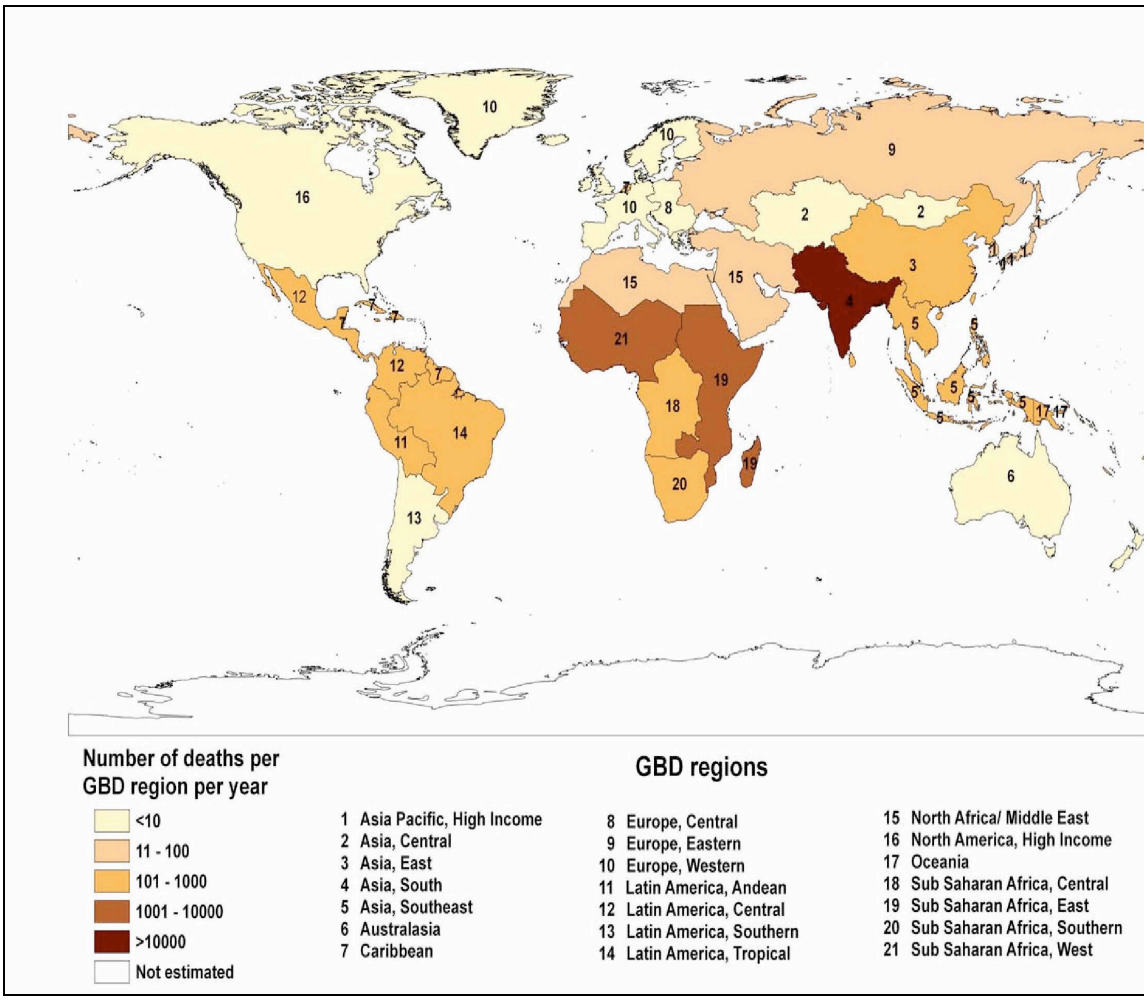


Figure 0.7 The global regional estimate of snakebite-induced deaths, the grouping of the countries was according to the Global Burden of Disease (GBD) (figure from; Kasturiratne et al., 2008).

1.4.4.1 Clinical features of snakebite envenoming

Mortality due to envenoming by venomous snakes is estimated to be 94,000–125,000 cases per year worldwide (Kasturiratne et al., 2008). Further, snakebites can cause long-term morbidity as limb necrosis may develop as a result of envenoming (Chippaux, 1998). The complexity of snake venom and the variation in its composition cause a wide range of post-envenomation symptoms. Clinically significant effects can be observed in envenomed patients that may cause potential morbidity and mortality. Venomous snakes that contain neurotoxins (Elapidae) can cause flaccid paralysis and systemic myolysis; others components (Viperidae) can act on the coagulation cascade and may lead to coagulopathy, haemorrhage, renal damage and failure, cardiotoxicity, and local tissue injury (White, 2005). These

symptoms are triggered by the action of venom toxins that have varying molecular targets and enzymatic activities (McCue, 2005).

1.4.4.2 Antivenom as treatment of envenoming

The only available specific treatment of snakebite envenoming is intravenous administration of animal-derived (mostly horse or sheep) antivenom (Gutiérrez et al., 2006). Many laboratories in different countries produce antivenoms using various methodologies. The production yields three different types of products depending on the nature of the active principle; antivenoms that consist of whole IgG molecules, antivenoms composed of $F(ab')_2$ fragments obtained by pepsin digestion of IgG, and a few laboratories produce Fab fragments after papain digestion of IgG (Gutiérrez et al., 2006). The restriction of antivenom efficacy to limited geographical and biological spectrum is explained by the large immunochemical diversity of snake venoms (Lalloo and Theakston, 2003). They are highly effective in the neutralisation of toxins responsible for systemic effects such as haemorrhage, coagulopathy, and haemodynamic disturbances. Likewise, neurotoxicity caused by postsynaptic neurotoxins in elapid envenomings can be reversed if treated early (Lalloo and Theakston, 2003). On the other hand, observations suggest that antivenoms are not very effective in the reversal of presynaptic induced neurotoxicity (Gutiérrez et al., 2006). The main problem associated with antivenom efficacy related to local tissue damage that is due to the extremely rapid development of local pathology rather than the lack of neutralizing antibodies in antivenoms (Gutiérrez et al., 1998). Antivenom safety is also of current concern since it often coincides with early and late adverse reactions (Theakston et al., 2003; Lalloo and Theakston, 2003).

As proteins (proteases and others) considered playing a major role in pathology initiation and progression, in both parasites and snake venom, the next few sections will briefly discuss some of the major proteins shared between parasites and snake venom.

1.4.5 Proteases and their role in parasitemia and envenoming

1.4.5.1 Proteases and trematodes (*S. mansoni* and *F. hepatica*)

S. mansoni and *F. hepatica* are trematodes that belong to the same phylum, Platyhelminthes. These digenetic trematodes have complex life strategies, involving changes between asexually and sexually reproducing life stages within different hosts; intermediate (mostly snails) and definitive (vertebrate species), respectively. To successfully complete this complex life cycle, these parasites produce molecules that play distinct and pivotal roles. For example, eicosanoids trigger local immunosuppression and vasodilatation and carbohydrates serve for molecular mimicry within infected hosts (Kasný et al., 2009). In addition, proteins in general and peptidases in particular, play a number of crucial roles during parasitism (Kasný et al., 2009). Peptidases produced by trematodes have been identified as essential enzymes in the immature (larval) and mature (adult) stages (Figure 8.1) (Cesari et al., 2000; Dvorak et al., 2005; Sajid et al., 2003). They are critical elements in many host-parasite interactions such as invasion, migration through tissue compartments, degradation of nutritional proteins (e.g., haemoglobin), immune evasion, and activation and modulation of inflammation (Caffrey et al., 2004; Delcroix et al., 2007; Donnelly et al., 2006; He et al., 2005; Koehler et al., 2007; McKerrow et al., 2006). For example, cercarial elastase a serine proteases is the key enzyme in infection establishment, metalloprotease in trematodes are essential for host immune system evasion and aspartic protease is used in hemoglobin digestion during feeding processes (Kasný et al. 2009; Ingram et al. 2003; Ingram et al. 2012; Caffrey et al. 2004; Silva et al. 2011; Koehler et al. 2007). The vital role of peptidases for trematode survival places these molecules as primary targets for the development of new drugs (e.g., cysteine peptidase inhibitor K11777) (Abdulla et al., 2007; McKerrow et al., 2006) or as constituents of molecular vaccines (e.g., cathepsins L1 and L2 of *F. hepatica*) (McManus and Dalton, 2006).

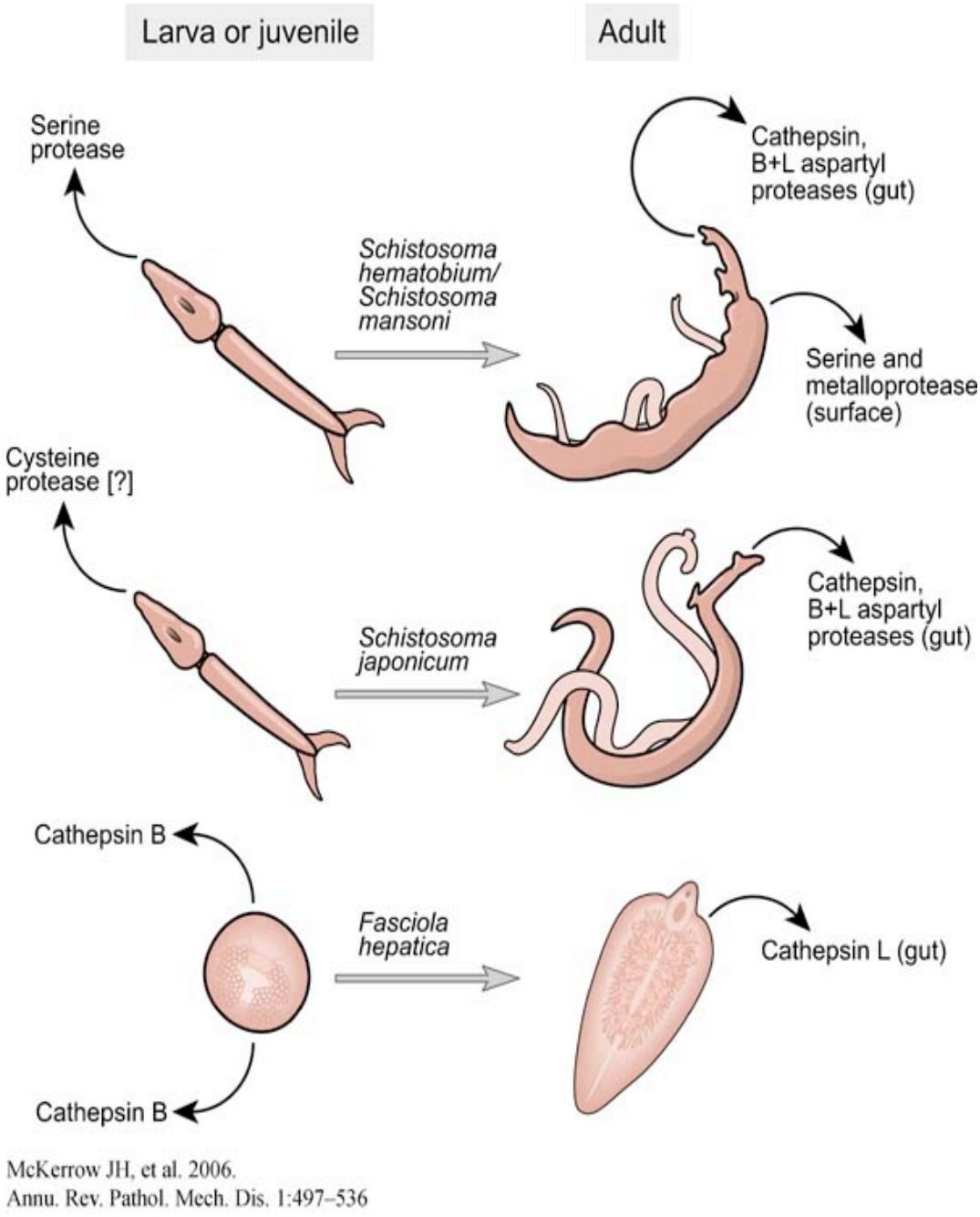


Figure 0.8 Comparison of protease release by larval versus adult helminth parasites. In larvae, proteases function primarily in tissue invasion or immune evasion. In adults, they function in gut degradation of host proteins, anticoagulation, and immune evasion (figure from; McKerrow et al. 2006).

1.4.5.2 Proteases and snake venom

Snake venoms are probably the most highly concentrated biological secretion found in vertebrates, and are complex mixtures of enzymatic and non-enzymatic proteins, peptidases and carbohydrates (Aird 2002). Venomous snake families produce pharmacologically active proteins: enzymes (phospholipase A_2 [PLA_2], l-amino acid oxidases [LAAOs], metallo- and serine proteases, phosphomonoesterases, phosphodiesterases, acetylcholinesterases, 5´nucleotidases, and hyaluronidases); nerve growth factors; compounds affecting blood coagulation by disturbing heamostatasis and induce bleeding; C-type lectins; activators and inhibitors of inflammation, septic shock, and tumour growth; myotoxins causing extensive damage to skeletal muscles, cardiotoxins; cytotoxins; platelet glycoproteins; natriuretic peptides; disintegrins; and three-finger toxins (Georgieva et al. 2008; Montecucco et al. 2008).

Despite the important role of snake venom proteases in immobilization and digestion of prey, they are responsible for various effects on biological functions after envenomation (e.g., haemorrhage, shock, or disorder of blood coagulation and nervous or muscular impulse transmission) (White, 2005; Lalloo et al., 1996).

The function and the role some of these shared proteins between parasites and snake venom will be described in the following sections.

1.4.5.3 Proteases shared between parasites and snake venom

1.4.5.3.1 Serine peptidases (SP)

1.4.5.3.1.1 Parasites (*S. mansoni* and *F. hepatica*) serine peptidases

Three main activity types of S1 family of peptidases have been described: i) chymotrypsin-like: peptidases expressing this activity prefer one of the hydrophobic amino acids at P1 position, phenylalanine over alanine by around 50,000 times; ii) trypsin-like: preference arginine or lysine at P1 position of the cleaved substrates; and iii) elastase-like: they generally prefer small aliphatic amino acids such as

alanine at P1 position (Kasný et al., 2009).

Schistosome cercarial elastase (CE) has a characteristic serine peptidase catalytic triad in the active site (His68/Asp126/ Ser218; numbering for SmCE) (Rawlings et al., 2008). It facilitates the degradation of various skin macromolecules including collagen, gelatin, keratin, fibronectin, laminin, and peptide backbone of proteoglycans or cell-cell contacts in the epidermis. The main interesting fact in CE is its ability to cleave another fundamental skin component, dermal elastin (Salter et al., 2000). Therefore, it is believed that elastases from cercarial acetabular glands play pivotal roles during cercarial host skin penetration. Additionally, schistosome CE may also help the cercariae evade the host immune response (Kasný et al., 2009).

The activity of another type of SP; kallikrein-like peptidase was purified from *S. mansoni* adults. The (66 kDa) peptidase was detected with d-Pro-Phe-Arg-p-nitroanilide substrate. The inhibition profile of *S. mansoni* kallikrein-like peptidase is characterized by common serine peptidase inhibitors, such as phenylmethylsulphonyl fluoride (PMSF), aprotinin, or soybean trypsin inhibitor (Carvalho et al., 1998). In addition it has shown the ability to cleave bradykinin and induce reduction of the arterial blood pressure of experimental animals (rats), possibly due to a peripheral vasodilatation effect which might reflect a similar effect in the natural host (Carvalho et al., 1998). Studies have demonstrated a variety of physiological functions for the enzyme such as processing of bioactive peptides, blood coagulation, and enhancement of glycosylation of IgE (Kasný et al., 2009).

1.4.5.3.1.2 Snake venom serine peptidases

Snake venom serine proteinases (SVSPs) are among the best-characterized venom enzymes affecting the haemostatic system. They belong to the trypsin family S1 of clan SA, the largest family of peptidases (Halfon and Craik, 1998). SVSPs can be classified into thrombin-like or kallikrein-like proteases. They affect various components in the blood coagulation cascade, on the fibrinolytic and kallikrein-kinin systems and on cells that in combination cause a major imbalance in the haemostatic system of their prey (Seegers and Ouyang, 1979; Markland, 1998; Pirkle, 1998). They are present in venoms of the families Viperidae, Crotalidae, Elapidae, and Colubridae. Snake venom enzymes characterized as serine proteinases are defined by

a common catalytic mechanism that includes a highly reactive serine residue that plays a key role in the formation of a transient acyl-enzyme complex, which is stabilized by the presence of histidine and aspartic acid residues within the active site (Barrett and Rawlings 1995).

The thrombin-like SVSPs are recognized by their ability to cleave fibrinogen at the arginine-lysine bonds on the α- and β-chain of fibrinogen releasing fibrinopeptides and consequently transforming fibrinogen into fibrin (Pirkle, 1998). On the other hand, kallikrein-like SVSPs share similarity with mammalian kallikrein enzymes in initiating the release of bradykinin by cleaving of kininogen proteolytically (Matusi et al., 2000). The release of bradykinin, known for its vasodilator nature, causes an increase in vascular permeability and hypotensive symptoms (Warrell 2010). Notwithstanding the high degree of mutual sequence identity, SVSPs show specificity toward a given macromolecular substrate (Markland, 1998).

1.4.5.3.2 Metalloproteases (MP)

1.4.5.3.2.1 Parasites (*S. mansoni* and *F. hepatica*) metalloproteases

In 2009, MEROPS the peptidases and peptidase inhibitors database 8.2 (http://merops.sanger.ac.uk) included 511 different metallopeptidase sequences of 15 clans and 54 families. Of these, ten various sequences of five trematode species were classified in seven clans and nine families (Rawlings et al., 2008).

1.4.5.3.2.2 Leucyl aminopeptidase (LAP)

Studies show that sequence identity of *S. mansoni*, *S. japonicum*, and *F. hepatica* LAPs is greater than 34% in multiple alignments. The highly conserved C-terminal domain, which is the active site motif 'NTDAEGR', was identified in all three amino acid sequences of *S. mansoni* LAP (SmLAP), *S. japonicum* LAP (SjLAP), and *F. hepatica* LAP (FhLAP) (Kim & Lipscomb 1993).

LAPs are broadly distributed cytosolic exopeptidases that possess six sub-units with 12 Zn^{+2} cations of around 56 kDa for SmLAP/SjLAP, and FhLAP (McCarthy et al. 2004; Rawlings et al. 2008). They cleave the leucine amino acid at P1 of the substrates, and not those with aspartate and glycine at P1 position.

Schistosome LAP is immunolocalized predominantly in the gastro-dermis and sub-tegument of adult parasites (McCarthy et al., 2004). Additionally, LAP activity was localized in *S. mansoni* eggs (Kasný et al. 2009). This suggests that schistosome LAP may participate in haemoglobin digestion and surface membrane re-modelling (McCarthy et al., 2004). LAP activities were also detected in the *S. mansoni* cercarial and schistosomular protein extracts. Furthermore, transcription analyses revealed significant expression of these peptidases by all developmental stages (Jolly et al. 2007; Auriault et al. 1982; Liu et al. 2006; McCarthy et al. 2004). Recombinant *F. hepatica* LAP (FhLAP) immunisation showed significant protection in immunized sheep (>89%) against metacercariae infection (Piacenza et al., 1999). It is also recognised as a potential immunodominant diagnostic marker, reacting with sera from fascioliasis patients.

1.4.5.3.2.3 Surface peptidase (Leishmanolysin)

Leishmanolysin (also called invadolysin), is a surface peptidase of the metallopeptidase M8 family found in *S. mansoni* proteome data (Silva et al., 2011). The conserved glutamate residue in the catalytic site acts in conjunction with a zinc ion to deprotonate and activate a water molecule. In turn, the activated water molecule acts as a nucleophile to attack the carbonyl of the peptide bond in a variety of substrates. These proteins are involved in different types of processes in Leishmania parasites, for example the inhibition or perturbations of host cell interactions and the degradation of the extracellular matrix (Fitzpatrick et al., 2009). These proteins may have similar activities in schistosomes. SmPepM8 (Smp_090100) is the second most abundant component of cercarial secretions (Silva et al., 2011). Other studies showed that SmPepM8 was also secreted from skin schistosomula, which provides insight on how it may contribute to tissue invasion by schistosomes and suggest this protein as a potential anti-parasitic target (Curwen et al., 2006; Fitzpatrick et al., 2009). Homology of this protein with a surface protease of Leishmania GP63 that contributes in surface remodelling of transforming larva against host complement may suggest a similar function in schistosomes (Dvorák et al. 2008). The presence of metalloprotease activity in early schistosomula may reflect the re-ordering of parasite surface as an adaptation process to intravascular

residence (Dvorák et al. 2008).

1.4.5.3.2.4 Snake venom metalloproteases

SVMPs are enzymes with multiple domains whose main toxic effects are due to disruption of the haemostatic system. SVMPs are classified within the M12 reprolysin family of metalloproteases and categorized according to their multidomain organization in classes PI to PIV (Bjarnason and Fox, 1994; Fox and Serrano, 2005). These enzymes are synthesized *in vivo* as multimodular proteins that comprise a signal peptide, a pro-domain, and a metalloprotease domain (class PI). Certain SVMPs also exhibit subsequent C-terminal domains, i.e., the disintegrin (class PII) or disintegrin-like domain (class PIII), the cysteine-rich domain (class PIII), and the C-type lectin-like domain (class PIV) or sub-unit. A recent update of the originally proposed SVMP classification scheme includes the following precursor classes: PIIa-PII precursors that in their mature form do not release the disintegrin domain; PIIb-PII precursors whose mature form comprises dimeric SVMPs; D-I-precursors that do not contain a metalloprotease domain; PIIIa-PIII precursors that release their disintegrin and cysteine-rich domains; PIIIb-PIII precursors that form dimeric SVMPs (Ramos and Selistre-de-Araujo, 2006; Fox and Serrano, 2005).

The zinc-binding motif, HEX box, is shared with ADAMs (for A Disintegrin And Metalloprotease) homologous proteins described in some organisms including mammalians. SVMPs and ADAMs belong to the reprolysin protein family and together with other protein families (astacins, serralysins, matrixins or matrix metalloproteases, snapalysins and leishmanolysins), form the clan of metzincins (Gomis-Rüth, 2003). The name metzincins is related to a conserved methionine residue in a β-turn downstream from the zinc-binding motif.

SVMP activity is associated with haemorrhage or the disruption of the haemostatic system, which are mainly mediated by the M domain proteolytic activity (Escalante et al., 2011). Haemorrhage is induced by SVMP interruption of the interactions between endothelial cells and the basement membrane through the degradation of endothelial cell membrane proteins (e.g., integrin, cadherin) and

basement membrane components (e.g., fibronectin, laminin, nidogen, type IV collagen) (Takeda et al., 2012; Gutiérrez and Rucavado, 2000). Furthermore, blood coagulation proteins (e.g., fibrinogen, factor X, prothrombin) are also targets of their proteolytic activities (figure 9.1).

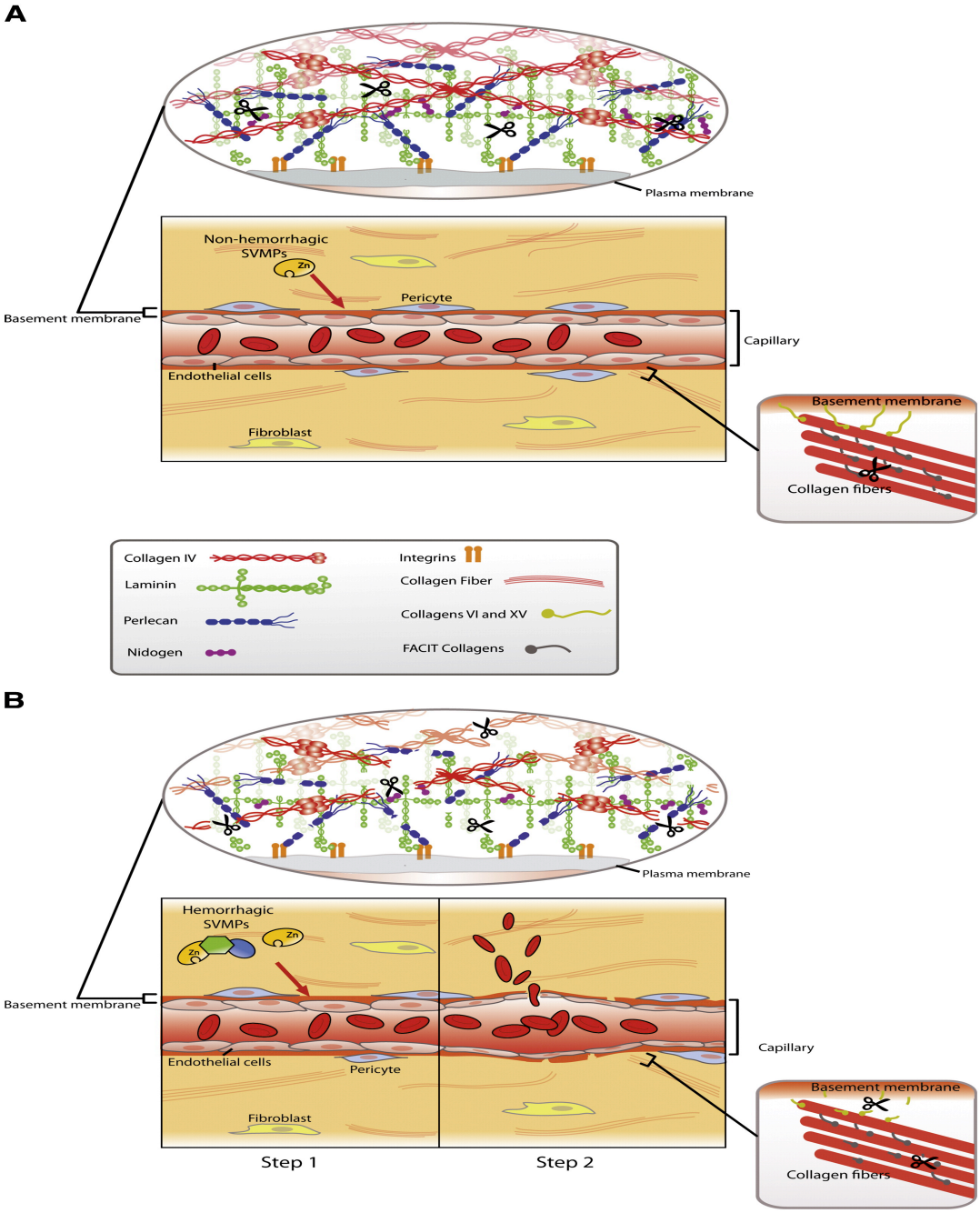


Figure 0.9 Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases. Non-hemorrhagic P-I SVMPs (A) hydrolyze some BM components, such as laminin, nidogen and some FACITs, whereas P-I or P-III hemorrhagic SVMPs (B) are capable of hydrolyzing, in addition to these substrates, other components which are likely to play a more critical role in capillary mechanical stability, i.e. types IV, VI and XV collagens, and perlecan. (figure from; Escalante et al. 2011).

1.4.5.3.3 Aspartic protease

1.4.5.3.3.1 Parasites (*S. mansoni* and *F. hepatica*) aspartic protease

In aspartic protease, an activated water molecule via the side chain of aspartate initiates the nucleophilic attack (Morales et al., 2004). Aspartic protease (cathepsin D, CD) sequences have been found in many trematodes. In schistosome, it has been localized in the gastrodermis of adult worms using immunohistochemical or molecular techniques (Schulmeister et al., 2005; Brindley et al., 2001). In addition to this, schistosome CD activity has been detected in eggs and miracidium of *S. japonicum* (Verity et al., 1999). CD is intimately involved in worm nutrition by facilitating degradation of host haemoglobin (Brindley et al., 2001; Delcroix et al., 2007; Caffrey et al., 2004). Moreover, schistosome CD may have a role in schistosome immune invasion as they can effectively cleave human IgG by removing Fc fragments, or degrade C3 factor of the complement (Verity et al., 2001).

1.4.5.3.3.2 Snake venom aspartic protease

Aspartic protease has been discovered in *E. ocellatus* venom gland transcriptome. Wagstaff and Harrison (2006) have provided evidence indicating that aspartic protease might be a secreted component of snake venom and therefore propose it as a new class of venom toxins. Sequence similarity has been demonstrated between venom aspartic protease and the sequence of mammalian CD, which is similar to renin and exhibits renin-like activity. The renin-angiotensin system is characterized by enzymatic activation of angiotensinogen to angiotensin I (ang-I), which is then hydrolysed by angiotensin-converting enzyme producing the vasoconstrictor ang-II (Pagliaro and Penna 2005; Lee et al. 1996). This might suggest a local hypertension induction by renin-like activity of *E. ocellatus* venom aspartic proteases in envenomed patients (Wagstaff and Harrison, 2006), as a consequence serving to exacerbate SVMP-induced tissue distraction; entry of venom aspartic proteins into the vascular system would also be expected to induce systemic hypertension (Wagstaff and Harrison, 2006).

1.4.5.4 Phospholipase A_2

1.4.5.4.1 Parasites (*S. mansoni* and *F. hepatica*) phospholipase A_2

Phospholipases are important enzymes that facilitate lipid metabolism; phospholipase A_2 (PLA_2) catalyses the specific hydrolysis of ester bonds linking the acyl chain to the C-2 position of 1,2-diacyl-3-*sn*-glycerophospholipids (Rogers et al., 1991). They include several unrelated protein families with common enzymatic activity. Two most important families are secreted and cytosolic phospholipases A_2. Other families include Ca^{2+} independent PLA_2 ($iPLA_2$) and lipoprotein-associated PLA_2s (lp-PLA_2), also known as platelet activating factor acetylhydrolase (PAF-AH) (Dennis 1994). PLA_2 is one of the most well studied phospholipid-degrading enzymes, partly because it is a major and important component of snake and bee venoms and of pancreatic digestive secretions. In parasites, the enzyme plays a potential role in lipid metabolism, membrane synthesis, remodelling, and/or separation and migration (e.g., tissue migration of L4 larva of nematodes) (Grieve et al., 2000; Hawn and Strand 1993). Previous studies have shown considerable homology between these enzymes from different organisms at both the amino acid and nucleotide levels, although they show little conformity in terms of their molecular size and their substrate and pH requirements (Rogers et al., 1991). Membrane-bound forms of PLA_2 are believed to be involved in membrane lipid homeostasis and are particularly active against highly cytotoxic phospholipid hydroperoxides that can be generated in membranes as a result of superoxide radical release from activated leukocytes (Rogers et al., 1991). Many studies have demonstrated that PLA_2 (Smp_166530.1) (lysosomal phospholipase A_2 or LCAT-like lysophospholipase), is present in trace amounts in *S. japonicum* and *S. mansoni* gut lumen, although it appears to be antigenic (Rogers et al., 1991; Nawaratna et al., 2011; Berriman et al., 2010).

1.4.5.4.2 Snake venom phospholipase A_2

Snake venoms classified in the Colubridae, Elapidae and Viperidae families comprise rich sources of PLA_2s (Gutiérrez and Lomonte, 2013). Structurally, snake venom PLA_2s are classified within groups I (elapid enzymes), found in mammalian

pancreas, and II (viperid enzymes) (Fry et al., 2008). The amino acid sequences and crystal structure of many snake venom PLA_2s have been reported, over 280 PLA_2 enzymes have been determined. Despite the differences in their pharmacological properties, structural characterization has revealed a relatively high identity (40–99%) in many of their sequences and a conserved structural scaffold (Kini, 2003). Nevertheless, these enzymes display an impressive array of pharmacological/toxicological activity effects including, in addition to pry digestion, pre- and post-synaptic neurotoxicity, myotoxicity, cardiotoxicity, coagulopathy, hypotension, haemolysis, and proinflammation (Kini, 2003; Montecucco et al., 2008). PLA_2 group I exhibit pre- or post-synaptic neurotoxicity. The pre-synaptic PLA_2 are responsible for the irreversible disruption of neurotransmitter release at the neuromuscular junction. On the other hand, post-synaptic neurotoxins competitively block acetylcholine receptors in the neuromuscular junction. This type of neurotoxicity can be reversed by rapid antivenom treatment (Lalloo and Theakston, 2003). PLA_2 myotoxins (group II) are more potent and fast-acting than non-enzymatic components of snake venom (Kini, 2003). They can induce myonecrosis or system myotoxicity. PLA_2-induced inflammatory reactions have been demonstrated; inflammatory cell infiltration promoted by PLA_2 isolated from *Naja naja atra* and phagocytic activity of macrophages stimulated by Asp49 and Lys 49 PLA_2s from *Bothrops asper* venom (Zuliani et al., 2005; Zhang and Gopalakrishnakone, 1999).

Shared proteins other than proteases like c-type lectin are found in parasites and snake venom and play a role in pathology caused by these pathogens. The next section is briefly summarises the function of some of these proteins in both parasites and snake venom.

1.4.5.5 Non-enzymatic proteins shared between parasites and snake venom

1.4.5.5.1 C-type lectin

1.4.5.5.1.1 Parasites (*S. mansoni* and *F. hepatica*) c-type lectin

C-type lectins (C-TLs) are a family of carbohydrate-binding proteins. They enable diverse biological processes including immune cell signalling and trafficking, activation of innate immunity, and venom-induced haemostasis (Weis et al., 1998). In helminth C-TLs share sequence and structural homology with mammalian immune cell lectins (Loukas and Maizels, 2000). Recently, they have been identified from nematode parasites, proposing clear roles for these proteins at the host–parasite interface, particularly in immune evasion.

Among the parasitic Platyhelminthes, no C-TL genes have yet been identified despite around 10,000 schistosome expressed sequence tags (ESTs) in the EST database. However, there is biochemical evidence for lectin expression in trematodes (Loukas and Maizels, 2000). There is positive evidence for the presence of surface/secreted lectins at most life cycle stages of the schistosome. Immune recognition of schistosome larvae by mollusc intermediate hosts is mediated by haemocyte surface lectins. Furthermore, a cDNA encoding a haemocyte C-TL homologous to selectins has been cloned from *B. glabrata* (Loukas and Maizels, 2000). In addition, acetabular glands of cercariae of the avian schistosome *Trichobilharzia* has been found to express activity of lectin on its surface. Lectin has been found to have specificity for tissue glycosaminoglycans, suggesting a role in recognition and/or penetration of host connective tissues.

Lectin-like activity in trematodes is not limited to larvae. Binding of a monoclonal antibody to human E-selectin to the surface of *S. mansoni* schistosomula was found to be inhibited in the presence of appropriate ligands for E-selectin (Loukas and Maizels, 2000). This finding may explain the ability of schistosomes to adsorb host molecules, for example antibodies, complement components, and major histocompatibility complex (MHC) class I antigens, on to their surface avoiding immune recognition (Loukas and Maizels, 2000).

1.4.5.5.1.2 Snake venom c-type lectin

In snake venom there are two types of C-TLs; the classic sugar binding C-TLs found in many animals and C-type lectin-like proteins (CLPs) found only in snake venom (Ogawa et al., 2005). Sugar binding C-TLs comprise a less toxic component of the venom and their role in venom is not fully understood (Weis et al., 1998). In contrast, CLPs of snake venom have a variety of biological properties, acting for example as anticoagulants, pro-coagulants, and agonists/antagonists of platelet activation. The structural and functional studies of the first identified CLP, factor IX/factor X-binding protein, show the ability of this protein to bind to the γ-carboxyglutamic acid domain of factors IX and X in Ca^{2+} (Atoda et al., 1994). Other unique CLPs have been also identified. The later show the ability to agonists and antagonists platelet receptors, platelet glycoprotein Ib and glycoproteins Ia/IIa and VI, collagen receptors and the following CLPs that modulate platelet function (Morita 2004; Huang et al. 1995).

As described in previous sections of this chapter, vectors like mosquitoes transmit many NTDs. Mosquitoes saliva contain a mixture of vasodilatory, antiplatelet, and anticlotting molecules to assists the process of taking blood meal (Calvo et al. 2006). Of these components, proteases and proteins that share nature with some snake venom proteins like serine proteases and C-TL have been identified. The next section describe briefly role of these proteins in mosquito saliva.

1.4.5.6 Mosquito *sialome*

Mosquito *sialome* is the set of mRNA and proteins expressed in the salivary glands, especially of mosquitoes. Salivary glands of mosquito contain hundreds of compounds that work as anti-haemostatic and immunomodulatory components aiding the finding and blood feeding from the vertebrate host. Only adult female mosquitoes are haematophagic, while both sexes take sugar meals.

Proteases have been identified from mosquito salivary glands. Of them, many serine proteases could be involved in specific host proteolytic events that could affect clotting or the complement cascade (Ribeiro et al. 2004; Francischetti et al. 2002). Other serine proteases are possibly involved in immunity, as they share a similarity with prophenoloxidase-activating enzymes responsible for the melanotic immune reactions of arthropods. In addition to serine protease, metalloprotease homologous to a *Drosophila* enzyme involved in remodelling of the salivary glands was also discovered in the salivary transcriptome of *An. gambiae* (Arcà et al. 2005).

The C-TL protein family is expressed in most mosquito sialotranscriptomes (Ribeiro et al. 2004; Arcà et al. 2005). This protein family is involved in immune recognition and in Plasmodium development in *Anopheles* mosquitoes (Ribeiro et al. 2007). The role of C-TLs in salivary immunity has not yet been demonstrated; nevertheless it is interesting that in snake venom this protein family has been employed in performing various unrelated functions such as an anticlotting, toxin, and platelet aggregation inducer (Morita 2004). Lectins may also play a role in the lectin pathway of complement activation (Ribeiro et al. 2007).

1.4.6 Aims

The vast literature discussing NTDs highlights the role of these diseases in the exacerbation of living conditions of the world's poorest populations. Epidemiological studies illustrate how populations suffer the burden of NTDs, where single individuals may be suffering from more than one NTD. Despite their variety, biological studies of NTD pathogens and factors show that several NTDs may use similar mechanisms in their pathology such as migration through tissue and cellular compartments; haemoglobin digestion and use of the vascular system as a carrier; the use of vectors as an infection-spreading tool; and inoculation in tissue or blood. They all evade immune system responses and cause necrosis and fibrosis to vital tissues and organs.

Looking at genomes, transcriptomes, and proteomes, these diverse pathogens appear to be utilizing similar molecules to perform similar biological mechanisms.

Therefore, it is of interest to ascertain whether a cross-cutting approach in research could facilitate a better understanding of these illnesses. Thus, the initial aim of this work was to examine molecular parallels of the mechanisms used by a variety of tropical disease pathogens, including parasites, snake venom toxins, and haematophagic parasite vectors, to access their host's blood stream.

To fulfil this target, *S. mansoni* and *F. hepatica* parasites and *An. gambiae* salivary glands were compared to major toxin (proteases and other proteins) in snake venom. Both *S. mansoni* and *F. hepatica* parasites have a migratory phase in which the parasite secretes proteolytic enzymes to break through tissue layers. In addition, adult *S. mansoni* live within the vascular system grazing on digested haemoglobin, avoiding haemostatic and immunological attack from blood components. Both parasites destroy the tissue surrounding the eggs of schistosomes and adult fasciola, and change the nature of the surrounding tissue structure causing necrosis and/or fibrosis. To do so, they use proteases that perform a similar function to their analogues in snake venom. Further, mosquito saliva has a wide range of proteins that help the insect to suck blood avoiding the danger of death by the bitten individual; these components have been found to share a similarity with snake venom.

In order to identify the molecular parallels between the proteases described above we designed a bioinformatics-led approach to investigate the transcriptomes of distinct *S. mansoni* life stages, *F. hepatica* adult, and mosquito sialome using publicly available data. This was followed by an investigation of the identified proteins expressed by these parasites, mosquitoes, and venom. After determining the shared molecules and their function in each pathogen, we subsequently aimed to examine the ability of antivenoms, used to prevent toxic effects of venom toxins, to neutralize homologous proteins in parasites and mosquito saliva using immunological tests.

The secondary aim of this research was to investigate opportunities of using homologous proteins in parasites, parasitic vectors, and snake venom as therapeutic applications. Molecules secreted and produced by these different pathogens have complex effects on human physiology, thus as an added benefit, these products may serve as the basis for novel therapeutic strategies. Consequently, we aimed to

construct chimeric epitopes of homologous snake venom (*E. ocellatus*) and mosquito salivary proteins as the primary vaccine and test whether boosting with proteins delivered by mosquito bites induces protective immunity against snakebites. This unique approach utilizes and exploits natural immunogens to stimulate sustained levels of protective immunity against vaccine-preventable diseases, with a particular focus on snakebite envenoming.

Chapter 2 Materials and Methods

1.5 Materials

1.5.1 Parasites

1.5.1.1 *Schistosoma mansoni* life cycle stages and preparation

All *S.mansoni* life cycle stages were homogenised samples (cercaria, cercarial transformation fluid (CTF), schistosomula, adult worm and soluble egg antigens (SEA)) and generously provided by Prof. Mike Doenhoff, University of Nottingham.

1.5.1.1.1 Reduced samples

50 µl of parasite homogenate (or 2.3µg/µl of total protein) per sample were solubilised in 250 µl of 0.02 M phosphate buffered saline (PBS) pH 7.2, incubated on ice for 30 minutes then centrifuged for 1 hour at 4 °C, 13,000 rpm. The supernatant was collected and aliquot to 50 µl in each tube. 2X protein loading buffer (PLOB), containing the reducing agent, β-mercaptoethanol, which act to further denature proteins by disrupting intra and inter molecular disulphide bridges, was added at volume of 12.5 µl for each aliquot, boiled for 5 minutes followed by quick cool down and re-centrifuged for 1min.

1.5.1.1.2 Non-reduced samples

The same protocol described above (reduced samples) was used to prepare non-reduced sample proteins. However, the loading buffer was prepared without reducing agent (5% β-mercaptoethanol).

1.5.1.1.3 Native proteins

50 µl of pre-prepared parasite homogenate were added to 12.5 µl of native protein loading buffer, boiled for 2 minutes followed by quick cool down and quick centrifugation at 4 °C, 13,000 rpm.

1.5.2 Fasciola hepatica

Fasciola hepatica parasites were a gift from Dr. James Lacourse. Flukes were stored at – 80 °C until prepared. The whole worm was homogenised in homogenisation buffer as described in Appendix I. The homogenates were prepared using the same preparation protocols used for *S.mansoni.*

1.5.3 *Anopheles gambiae* salivary glands

Females *An. gambiae* were raised and kept in Liverpool School of Tropical Medicine insectary. Salivary glands were dissected in 0.02 M PBS pH 7.2 and stored at – 80 °C. The pooled salivary glands were homogenised using tissue homogenizer on ice, vortex for 30 seconds then centrifuged for 30 minutes at 4°C, 13,000 rpm. The resulting supernatant was aliquoted and stored at – 20 °C. The preparation of the sample for electrophoresis and immunoblotting was as described above for the Schistosoma life cycle stages.

1.5.4 Snake venom

All venoms used in this study were extracted from snakes maintained in herpetarium at the Alistair Reid Venom Research Unit in Liverpool School of Tropical Medicine under special dietary and climatic conditions. Paul Rowley and Dr Rob Harrison conducted the venom extractions.

The following snake venoms were used in this study: *Echis ocellatus*, *Echis pyramidum leakey*, *Bitis arietans*, *Naja haje*, *Naja nigricollis*, *Naja pallida*, *Dendroaspis angusticeps, Acanthophis antarcticus*. Lyophilized venom samples were reconstituted at a concentration of 10mg/ml in 1X phosphate-buffer (1XPBS) and stored at -80^{o}C. Before venom protein analysis on SDS-PAGE gel, samples were diluted to a final concentration of 1mg/ml in 2X PLOB. For non-reduced samples, non-reduced 2X PLOB was added and both (reduced and non-reduced samples) prepared as described above.

All buffers and stock solutions used throughout the course of this experimental work can be found in Appendix 1.

1.5.5 Human sera and immunoglobulins

In chapter 5 of this study we tested immune-cross reactivity between snake venom and purified human immunoglobulins; Malawian malaria patients IgGs, IgGs and IgM purified for therapeutic reasons, GAMMAGARDS and PENTAGARDS respectively, and whole serum collected from patients with schistosomiasis and filariasis infection.

Purified immunoglobulins; Malawian malaria patients IgGs, GAMMAGARDS (IVIgG) and PENTAGARDS (IVIgM), were gifted by Dr Richard Pleass.

IVIgG purified from malaria infected Malawians was obtained from blood donated for erythrocyte transfusions by 834 Malawian adults who were seronegative for anti-HIV antibodies (ELISA. Wellcome). Plasma was air-freighted on dry ice to the Central Laboratory of the Swiss Red Cross Blood Transfusion Service in Berne, Switzerland. Samples negative for both HBsAg (Ausria II,Abbott) and anti-H IV antibodies (anti H IV- 1, EIA, Abbott; anti HIV- I + 2, EIA, Abbott) were pooled and submitted to the commercial process of immunoglobulin extraction. The process includes cold ethanol fractionation and pH4 treatment, both procedures shown to remove and or inactivate viruses (Taylor et al. 1992)

GAMMAGARDS (IVIgG) was processed from the pooled plasma of 3000 healthy donors and provided as freeze-dried preparation (Baxter Healthcare Corporation, USA) (Mekhaiel, Czajkowsky, et al. 2011). Similar, PENTAGARDS (IVIgM) prepared as pooled polyreactive normal IgM that contains >90% i.v. IgM (IVIgM) from plasma of >2500 healthy donors and provided as freez-dried preparation (Hurez et al. 1997; Kaveri et al. 2012). Prior experiments, the IVIgs were reconstituted in PBS to the desired dilution according to the experiment and kept sterile at 4°C.

Schistosomiasis patients' sera collected by Dr Russell Stothard group. Schistosomiasis patients' sera were collected from two villages in Uganda; one has a high prevalence of infection (Bugoto) and the other with low prevalence (Lawanika) on two separate collections. The first was baseline collection before treatment and

the other one-year after treatment. These samples were a part of ongoing monitoring and evaluations of the Schistosomiasis Control Initiative (SCI) program in Uganda that started on 2003(Koukounari et al. 2006; Zhang et al. 2007; Balen et al. 2006).

Dr Mark Taylor's group in Liverpool School of Tropical Medicine generously provided filariasis patients' sera, symptomatic and asymptomatic (Hoerauf et al. 2003). The samples were collected during a study held in Ghana. In an endemic area for lymphatic filariasis in the Western Region of Ghana at which mass treatment with drugs had not started, 78 men and 15 women aged 18–55 years with a minimum microfilaremia of 40 mf/ml were selected in six villages. The selected villages have no other human filarial species endemic. Exclusion criteria were applied as follow; abnormal hepatic and renal profiles (alanine aminotransferase >30 U/l; c-glutamyl-transpeptidase >28 U/l; creatinine >1.2 mg/ 100 ml) measured by dip-stick chemistry (Reflotron, Roche Diagnostics, Mannheim, Germany), body weight <40 kg, alcohol or drug abuse, chronic medication, pregnancy or breast feeding in females, and the mental inability to understand the explanations of the study design. All participants gave informed consent (Hoerauf et al. 2003).

1.6 Methods

1.6.1 SDS-PAGE gel preparation and electrophoresis

Samples homogenates were size-separated by SDS-PAGE (15%) according to manufacturer's recommendations (BioRad, UK). 1mm glass plates were assembled using the mini-Protean electrophoresis system (BioRad, UK). 1mm 15% polyacrylamide resolving gels (for 4 gels: 7.5 ml dH_2O, 5ml 1.5M Tris pH 8.8, 7.5ml 40% Bis Acrylamide, 200µl 10% SDS, 120µl 10% ammonium persulphate (APS) and 14µl TEMED) were prepared, poured into the glass plates and overlaid with dH_2O in order to remove any bubbles and prevent drying. The gels were allowed to polymerize for 45 min. Following polymerization, dH_2O was removed and stacking gel mixture (for 4 gels: 5ml dH_2O, 2ml 0.5M Tris pH 6.8, 350µl 40% Bis Acrylamide, 30µl 10% APS and 5µl TEMED) was loaded on top of polymerized resolving gel and a 15-well 1mm comb was inserted between the plates. Again, the

stacking gel was allowed to polymerise for an additional 45 min. Electrophoresis was carried out using a mini-Protean cell (BioRad, UK) at 200V for 45 min to 1 hour in 1X Tris-glycine-SDS (TGS) running buffer. A standard broad range molecular weight protein marker ranging between 10 to 225kDa (Promega, UK) (5µl) was run alongside 5µl of samples used in this work in order to give an indication of the molecular weight of the proteins in the samples. Separated samples on 15 % gels were stained with Coomassie Blue R-250 overnight and destained with Coomassie destain solution until clear.

1.6.2 Immunoblotting

After protein concentration optimization was done, new SDS-PAGE gels prepared loaded with 5µl of each sample and electro-blotted to 0.45µm nitrocellulose membranes as described in the manufacturer's protocols (BioRad, UK). Immunoblotting was used to detect specific proteins in parasites, mosquitoes' salivary glands and venom that were immunoreactive to antivenoms, toxin-specific antibodies and human sera and immunoglobulins. Following separation of proteins on 1D SDS-PAGE (conducted as described in the previous section), the polyacrylamide gel was sandwiched together with a 0.45 µm nitrocellulose membrane (BioRad, UK) between two double layers of filter paper and foam padding. Then the sandwich was inserted into a cassette and placed into an immunobloting transfer tank (BioRad, UK) filled with immunotransfer buffer (2.03g Tris, 14.26g glycine, 800ml H_2O and 200ml methanol). After one hour of electro-transfer at 100V with continuous mixing by a magnetic stirrer, the transfer of proteins from the SDS-PAGE gel onto the nitrocellulose membrane was complete. During electo-transfer an ice block was inserted into the transfer tank to prevent the buffer from overheating during the process.

Bands on the nitrocellulose membrane were visualized using 1X Ponceau S dye to check the efficiency of electro-transfer and to enable labeling of markers and sample well lanes. After that, membranes were thoroughly rinsed in 1X Tris-buffered saline Tween-20 (TBST) to remove the Ponceau S dye. Following washing steps, the membranes were blocked by overnight incubation in blocking buffer (5 % non-fat milk in TBST) to reduce non-specific protein binding. The following day the

strips were washed 3 times in TBST over 60 minutes and incubated with Blocking buffer (details in Appendix I) for 3 hours and then incubated overnight with the test antibodies (variety of polyclonal and monoclonal snake antivenoms, toxin specific IgGs and human sera and immunoglobulins) (table 4.1) at concentration ranging from 1:1000 and 1:5000 for antivenoms and 1:100 to 1:200 for the rest in blocking buffer. Blots were washed with TBST as described above and incubated for 1 hour with secondary antibodies (rabbit anti-horse, donkey anti-sheep, goat anti-mouse and anti-human IgG) (1:1000 dilution) coupled to horseradish peroxidase (HPR). Visualization of the results was performed using a 3,3'-Diaminobenzidine peroxidase (DAB) peroxidase substrate (Sigma, UK) after washing with TBST.

1.6.3 Mass spectrometry

1.6.3.1 In-gel trypsin digestion

Protein bands from the immunocross-reacteive proteins from the parasites and mosquito samples were excised from one-dimensional SDS-PAGE gels. The bands were destained and in-gel trypsin-digested as described by Hayter *et al* (Hayter et al. 2003). The protein plug was excised from 1D SDS-PAGE gel using fine scalpel and transferred to a 1.5 ml microcentrifuge tube. To destain the plug and remove all traces of Coomassie stain, 20-50µl of distaining solution containing 50 mM Ammonium bicarbonate/ 50% (v/v) acetonitrile (AmBic/ACN) was added and incubated for 10 minutes at 37°C. This step was repeated by replacing the solution with a fresh one until no stain remains in the plug and the gel is transparent. Once destained, 20µl of 10mM dithiotheritol (DTT) reducing agent was added to each tube and incubated at 37 °C. After 30 minutes, DTT was removed and the samples were alkylated by incubating them in 20µl of 55mM iodoacetamide (IAN) at 37 °C for 30 minutes in the dark. Following that, the plugs were dehydrated using 10µl of 100% ACN, and the incubation continued for 15 minutes. After dehydration, the supernatant was removed from the plug (should turn white) and the remaining solvent was allowed to air dry at 37°C. Once dry, 10µl of 1:10 trypsin (stock prepared by re-suspending 25µg proteomics grade trypsin (Sigma, UK) in 50mM acetic acid) in 50mM ammonium bicarbonate/ 50% ACN (giving a final concentration of 0.1µg/µl) was added and incubated for 1 hour at 37°C. The digestion was allowed to continue overnight after the addition of 10µl of the trypsin

solution. The next day, the reaction was halted by the addition of 2µl of 2.6M formic acid. The digested bands were then dried in a SpeedVac centrifuge and stored at -20°C. Prior to analysis, the bands were rehydrated in 50µl HPLC-grade water, sonicated for 15 minutes and centrifuged for 20 minutes at 13,000 rpm.

1.6.3.2 HPLC- tandem mass spectrometry (HPLC-MS/MS) and protein identification using bioinformatics tools

In order to identify parasite and mosquito salivary gland proteins that showed immunocross-reactivity to anti-venom, homogenated samples (5mg/ml solution in 0.1% formic acid mobile phase buffer) were centrifuged for 20 minutes at 13,000 rpm. The LC-MS/MS analysis was performed as described by Currier *et al* (Currier et al. 2010). Using Ultimate 3000 system (Dionex, UK), the samples were fractioned in the first diminution using ion exchange 2mm x 50mm ProPac ® SCX-10G column over a gradient (600-900mM NaCl in 0.1% formic acid, pH 2.3) at a flow rate of 60µl/min. The generated fractions (20 per sample) were subjected to second dimension fractionation using a 300µm I.D> x 15 cm capillary LC ion-exchange column packed with 3µm PepMap™ C18 stationary phase. The gradient used for this stage was 2-90% acetonitrile in 0.1% formic acid over 50 min at a flow rate of 300nl/min. Peptides eluted from the above process were analysed on an LCQ Deca XP Plus Mass spectrometer (ThermoFisher, UK), operating on a 'triple play' mode (zoom scan followed by MS/MS).

The identification of proteins was performed using Proteome Discoverer 1.0.0 (ThermoScientific) software, by screening LC-MS sequence data against Uni-prot database incorporating both Sequest (ThermoScientific (Yates III et al. 1996) and Mascot (Matrix Science Ltd, UK (Perkins et al. 1999) search algorithms. For the analysis, 1.5Da parent mass tolerance was used, allowing for 1 missed cleavage. For modifications, carbamidomethylation of cysteine was fixed and oxidation of methionine was variable. The degree of confident was optimized as follow, high confidence peptide matches for Sequest were filtered with an XCorr cut-off (+1 > 1.5, +2 > 2 and +3 > 2.5) and for Mascot, an Exp value of less than 0.01 specified a confident peptide match (Currier et al. 2010).

1.6.4 Enzyme-Linked Immunosorbent Assay (ELISA)

1.6.4.1 End-point ELISA titration

Assays were prepared using 100ng of venom from *Echis pyramidum leakey* per well. 30µl diluted venom (1mg/ml in PBS) was added to 30ml of coating buffer (1.59g Sodium carbonate Na_2CO_3, 2.93g Sodium bicarbonate $NaHCO_3$, 0.2g Sodium azide NaN_3, 1L H_2O at pH 9.6) to give a final concentration of 1ng/µl then loaded to a 96 well ELISA plate. The plate was then wrapped in cling film and incubated overnight at 4°C. The following day, the plate was washed with TBST six times then non-specific protein binding blocked with 100 µl 5% non-fat milk (blotto) (diluted with TBST – 0.01M Tris- HCl, pH 8.5; 0.15M NaCl; 1% Tween 20) for 3h at room temperature (RT) wrapped in cling film. After 3h the plate washed six times in TBST and incubated with the test antibodies (human immunoglobulins and parasitic patients sera (purified human immunoglobulins; Malawian malaria patients IgGs, IgGs and IgM purified for therapeutic reasons, GAMMAGARDS and PENTAGARDS respectively, and serum collected from patients with schistosomiasis and filariasis infection) prepared as follows: each well of the the plate was loaded with 100µl blotto except for the first wells (first column). The first column was loaded with 150µl of 1:10 diluted primary antibody followed by 1:2 serial dilutions the method was repeated across the wells of the plate until the one before the last, where 50µl is removed and discarded. The last row of the plate was left with blotto only as a control. Then the plate was wrapped in cling film and incubated overnight at 4°C. The next day the plate was washed again in TBST as described previously and incubated in horseradish peroxidise-conjugated goat anti-human IgG (1:1000; Sigma, UK) for 3 h at RT. Results were visualized by addition of substrate (0.2% 2,2/-azino-bis (2-ethylbenzthiazoline-6-sulphonic acid) in citrate buffer, pH 4.0 containing 0.015% hydrogen peroxide; Sigma, UK) and measurement of optical density (OD) at 405nm. End point titres were determined by the immunoglobulin/ sera titre that exhibited OD readings greater than three standard deviations of the control reading.

1.6.4.2 Relative avidity ELISA

Plates were coated with *E. p.leakeyi* venom as described above and incubated overnight at 4°C with 1:10 for immunoglobulins and 1:50 for sera (the avidity titres were optimized according to end-point titres and amount of sera available). After washing with TBST, 0.1ml of the chaotrope, ammonium thiocyanate (that disrupts protein-protein bonds and therefore used here a measure of the strength of antibody-antigen binding) was added to the wells in a range of concentrations (0–8 M) for 15min followed by washing in TBST prior to the addition of the secondary antibody. Plates were washed, and all subsequent steps were the same as the end-point ELISA. Relative avidity was expressed as the avidity-index of ammonium thiocyanate concentration at which 50% of the bound antibodies are eluted off.

1.6.5 PNGase F enzyme Deglycosylated venom blots

In eukaryotic cells glycosylation is a major post-translational modification (Bekri, 2006). There are several pathways for protein modification with sugars (glycans), the most common being the addition of glycans to an asparagine (*N*-linked) or to a serine or threonine (*O*-linked) residue in the secretory compartment (ER and Golgi).

This protocol describes the enzymatic removal of glycans from venom protein using PNGase F (BioLabs, UK). This experiment was done to determine if post-translation modification of venoms (*Echis ocellatus*, *Echis pyramidum leakey*, *Bitis arietans*, *Naja haje*, *Naja nigricollis*, *Naja pallida*, *Dendroaspis angusticeps, Acanthophis antarcticus*) is affecting the cross reactivity with human sera and immunoglobulin. PNGase F is able to remove most *N*-glycans, while the enzymes in the Protein Deglycosylation Mix [PNGase F, *O*-Glycosidase (Endo-α-N-Acetylgalactosaminidase) and other enzymes] remove *N*-glycans, short *O*-glycans, and certain long chain *O*-glycans.

The venom-deglycosylation enzyme mixture was prepared as described by manufacturer (BioLabs, UK). The following components were mixed in 0.5ml eppendorf tube: 14µl venom (140µg) was mixed with 2µl 10X denaturing buffer and

4µl dH_20. The mixture was prepared and the entire solution components were kept on ice. The mixture was then boiled at 100°C for 10min. After that 4µl 10X G7 buffer, 4µl 10% NP-40, 1µl PNGase F and 1µl dH_20 were added to the mixture. Then the mixture was incubated for 1hr at 37°C. At the end of incubation time 100µl of reduced 2X PLOB (40µl 2X PLOB and 60µl PBS) was add to the 30µl deglycosylated venom sample and boiled for 2mins. 10µl of each sample were loaded on a 15% SDS-PAGE gel were the first lane was loaded with 10µl of protein marker.

1.6.6 Ethical declaration

All animal experimentation conducted during the course of this work was undertaken using standard protocols approved by the Liverpool School of Tropical Medicine and University of Liverpool Animal Welfare Committee and performed with the approval of the UK Home Office under project license #40/3216.

For filariasis patients' sera, the Ethical Committee of the School of Medical Sciences of the University of Science and Technology, Kumasi, Ghana approved the study. Study procedures were in accordance with the Helsinki Declaration of 1975 (as revised 1983 and 2000). Pfizer Inc. (Karlsruhe, Germany) provided Vibramycin^{Ò} tabs (Hoerauf et al. 2003).

For schistosomiasis patients, ethical clearance was obtained from the Ugandan National Council of Science and Technology and Imperial College London. For ethical reasons, it was not appropriate to include any untreated control groups in the study design.

THE REPUBLIC OF UGANDA

OFFICE OF THE PRESIDENT

PARLIAMENT BUILDINGS P.O. BOX 7168 KAMPALA, TELEPHONES : 254881/6, 343934, 343934, 343926, 343943, 233717, 344026, 230048, FAX : 235459/256143

ADM 154/212/01

October 13, 2009

The Resident District Commissioner
Mayuge District

This is to introduce to you **Dr. Stothard John Russell** as a Researcher who will be carrying out a research entitled **"Control of intestinal schistosomiasis (bilharzia) and molecular epidemiology of schistosoma mansoni in Ugandan infants and preschool children"** for a period of **04 (four) years** in your district.

He has undergone the necessary clearance to carry out the said project.

Please render him the necessary assistance.

Alenga Rose
FOR: SECRETARY, OFFICE OF THE PRESIDENT

Chapter 3 Bioinformatics analysis to identify shared molecules *in* *Schistosoma mansoni, Fasciola hepatica* and snake venom

3.1 Introduction

Understanding the molecular functions used by microorganisms and identifying new therapeutic targets has been increasingly accomplished and rationalized using genomic and bioinformatics tools. In this study, we focus on identifying similarities between two kinds of proteases—those that are used by parasites to migrate through host tissue compartments, evade the immune system, digest host blood and disturb the infected individual's haemostatic mechanism, and those present in snake venom that lead to the victim's local and systemic pathology.

Schistosome parasites use several serine proteases to facilitate vital biological function. They use cercarial elastase, a chymotrypsin serine protease, as a major invasive larval protease during host skin penetration (Ingram et al. 2012; Salter et al. 2002). Another type of serine protease was identified in schistosome cercaria and adult stage, the SmSp1, a kallikrein-like serine peptidase (Cocude et al. 1997; El-Ansary 2003; Kasný et al. 2009). Kallikrein-like peptidases participate in a variety of physiological functions such as processing of bioactive peptides or blood coagulation (Kasný et al. 2009). In schistosome SmSp1 was localized on the surface of male worms and in parenchyma of both sexes suggesting a role of SmSP1 is in modulation/evasion of the host immune response (Cocude et al. 1997; Kasný et al. 2009). In parallel, snake venom serine proteases (SVSP) play a pivotal role in affecting the haemostatic system (Serrano and Maroun, 2005). They act on a variation of coagulation cascade components, on the fibrinolytic and kallikrein–kinin systems and on cells to cause an imbalance of the haemostatic system of the prey (Serrano et al. 1998).

Metalloprotease is another protease utilized in several vital processes in parasitism and envenomation. In trematode parasites (schistosome and fasciola) metalloprotease (leucyl aminopeptidase and dipeptidyl peptidase III) have potentially crucial roles in parasite nutrition (hemoglobin digestion) and maintenance of tegumental structure (dismantling, degradation and turnover of the protein components of the membrane) (Day and Chen, 1998; Kasný et al., 2009; McCarthy et al., 2004). It's expressed in the early invasive stages of the parasite as well as adult stages and egg (McCarthy et al. 2004; Kasný et al. 2009). On the other hand, snake venom metalloproteases (SVMP) induce hemorrhage or disruption of the hemostatic

system, which are primarily mediated by the proteolytic activity of the M domain (Takeda et al. 2012). Hemorrhage is induced by disturbing the interactions between endothelial cells and the basement membrane through the degradation of endothelial cell membrane proteins and basement membrane components (Markland and Swenson 2013). While targeting blood coagulation proteins disturbs the hemostatic system (Takeda et al. 2012; Morita and Iwanaga 1981).

Other peptidases and enzymes like aspartic protease (Koehler et al. 2007; Schulmeister et al. 2005; Wagstaff & Harrison 2006) and phoshpholipase A_2 (Rogers et al. 1991; Kini 2003) also found to have a pivotal role in essential and ingenious mechanisms that insure sustainability and succession of infection/envenomation.

Given the potential similarities between proteases found in parasites and those comprise major toxin proteins in snake venom and to improved understanding of molecules that are critical to success of parasitism/envenomation, the transcriptomic data of *S. mansoni*, *F. hepatica* and snake venom (*Echis ocellatus*) was bioinformatically investigated. The assembled contig library of each life stage of *S. mansoni* (cercaria (SCAP), 7-day schistosomula (SLAP-7), lung-stage schistosomula (SLAP), 21- and 28-day adult (SWAP-21, SWAP-28), and egg stages (SEA)) and of *F.hepatica* adult stage (FAP) that involved in migrating or causing pathological damage to the host was selected for this study. These stages were selected as they use at least one type of protease in their biological functions such as migration for cercaria and schistosomula and blood digestion for adults. Transcriptomic data has provided opportunities for a more rational approach to expanding our perspective of parasite biology and gaining a better understanding of processes such as host-parasite interaction in order to propose drug targets and vaccine candidates for parasitic diseases and to study evolution and comparative genomics, transcriptomics and proteomics (DeMarco & Verjovski-Almeida 2009; Protasio et al. 2012; Nahum et al. 2012; Wilson et al. 2007). *In silico* and bioinformatics studies have revealed the possibility of identifying important novel proteins and proteases. Recent technological improvements in cDNA sequencing using next generation sequencing (NGS) platforms have permitted the development of various new strategies for high-throughput automated sampling of DNA and cDNA. These developments have supported an exponential increase in the number of

sequences available in public databases, thus increasing the yield of biological information that facilitates the identification of molecules playing a key role in biological pathways or processes that could become targets for new parasitological therapy (Young et al. 2010).

Briefly, expressed sequence tags (ESTs) assembled as contigs for the different *S. mansoni* life stages and *F. hepatica* (SCAP, SLAP-7, SLAP, SWAP-21, SWAP-28, SEA and FAP) were downloaded from schistodb website (ftp://ftp.sanger.ac.uk/pub/pathogens/maa/S.mansoni/ESTs/) and adult-stage *F. hepatica* (FAP) from (ftp://ftp.sanger.ac.uk/pub4/pathogens/Fasciola/hepatica/ESTs/) and compared the to available transcriptomic data for *E. ocellatus* (Eo-db), developed at the Alistair Reid Venom Research Unit (Wagstaff and Harrison 2006). First, the databases were blastp-searched against the *Echis ocellatus* venom database (Eo-db) to explore the presence of similarity and conservation between transcriptome of parasites and snake venom. Next, ESTs encoding parasite proteins that shared similarity with snake venom were analysed using the multiple alignment program Constraint-based Multiple Protein Alignment Tool (COBALT) available at (http://www.ncbi.nlm.nih.gov/tools/cobalt/). Then, functional annotations of the parasites and snake venom databases were compared to gain more information for understanding the functions of transcriptomic data of the parasites and venom using molecular function Gene Ontology (GO) terms. Gene Ontology (GO) is a controlled vocabulary of terms for describing behavior of genes and their products (Harris et al. 2004), which is valuable to measure gene functional similarity. The data were analyzed using Blast2GO software. The Blast2GO suite is a comprehensive bioinformatics tool for functional annotation of sequences and data mining on the resulting annotations, mainly based on the gene ontology (GO) vocabulary (Conesa & Götz 2008).

1.7 Methods

1.7.1 Data

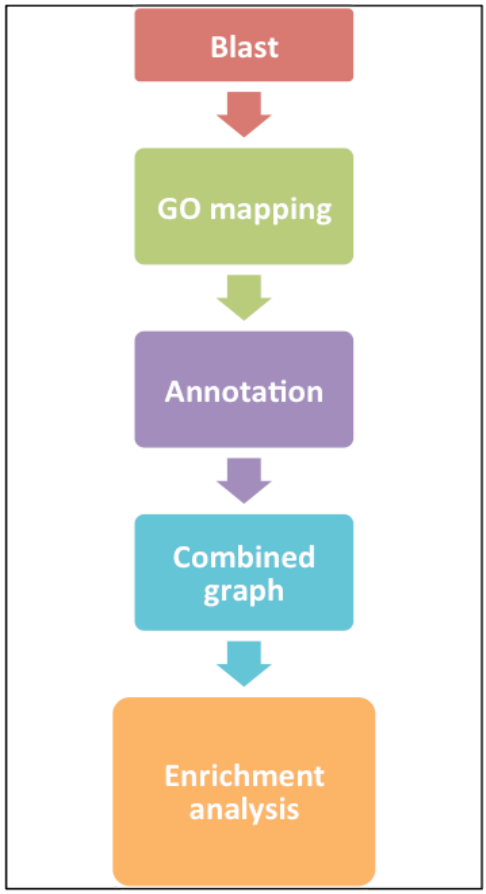
A total of 15,240 *S. mansoni* contigs assembled in FASTA format sequence data. The data was separated according to life stage (cercaria (SCAP), 7-day schistosomula (SLAP-7), SLAP, 21- and 28-day adult (SWAP-21, SWAP-28) and soluble egg antigen (SEA)). These assembled contiguous sequences are available on the schistodb website (ftp://ftp.sanger.ac.uk/pub/pathogens/maa/S.mansoni/ESTs/). Further, to identify putative homologues between trematodes (*S. mansoni* and *F. hepatica*) and snake venom proteases, 3332 EST sequences from adult-stage *F. hepatica* (FAP) (ftp://ftp.sanger.ac.uk/pub4/pathogens/Fasciola/hepatica/ESTs/) were also downloaded as FASTA file. The parasites databases were pairwise blasted against *E. ocellatus* (Eo-db), developed at the Alistair Reid Venom Research Unit (Wagstaff and Harrison 2006). BLAST search of parasites contiguous sequences were undertaken using an Intel, dual 2.8GHz RedHat Enterprise Linux 9.0 workstation running Biolinux 6.0 (available at http://envgen.nox.ac.uk) gainst *E. ocellatus* (Eo-db) data. BLASTp was performed using the command: (blastall –i schistosma_proteases.fasta –d echis_ocellatus_data.fasta –p blastp –o schistosoma_vs_echis.txt –v 10 –b 10) at e-value of $1e^{-05}$. Retrieved parasites sequences were aligned to parallels from snake venom databases using the CLUSTAL alignment tool in Jalview a free program for multiple sequence alignment editing, visualization and analysis (available at; http://www.jalview.org) (Waterhouse et al. 2009).

1.7.2 Blast2GO software

The functional annotation of the parasites and snake venom transcriptome were compared using Blast2GO software. Blast2GO software suite is a comprehensive and integrated bioinformatics tool for the automatic, high-throughput, functional annotation of DNA or protein sequences and data mining for the resulting annotation, on the basis of GO vocabulary (Götz et al. 2008). GO annotation in this software proceeds through BLAST searches against sequence databases, mapping of homologue sequences to GO terms and final GO term

assignment using the Blast2GO annotation rule (Conesa and Götz, 2008; Götz et al., 2008). Annotation algorithm used by this software took multiple parameters into account such as sequence similarity, BLAST HSP (highest scoring pair) length and e-values, the GO hierarchical structure and GO term evidence codes (Götz et al. 2008). The parameters and the steps used by the program in this analysis are described in Figure 3.1.

a



b

BLAST	Blast DB	swissprot
	Number of blast hits	20
	Blast expected value	1.0 E-3
	Blast program	blastx
	Blast mode	Qblast-NCB
	HSP length cutoff	33
Annotation	E-value hit filter	1.0 E-6
	Annotation cutoff	55
	GO weight	5
	HSP-hit coverage cutoff	0
Combined graph	Tree type	function
	Seq filter	0
	Node information	node score
	Mode of graph-colouring	by score
	Score alpha	0.6
	Node score filter	0

Figure 0.1 a. Blast2GO project phases; b. parameters used in B2GO analysis.

1.8 Results

1.8.1 BLAST and the alignment of *S. mansoni* and *F. hepatica* ESTs with snake venom ESTs

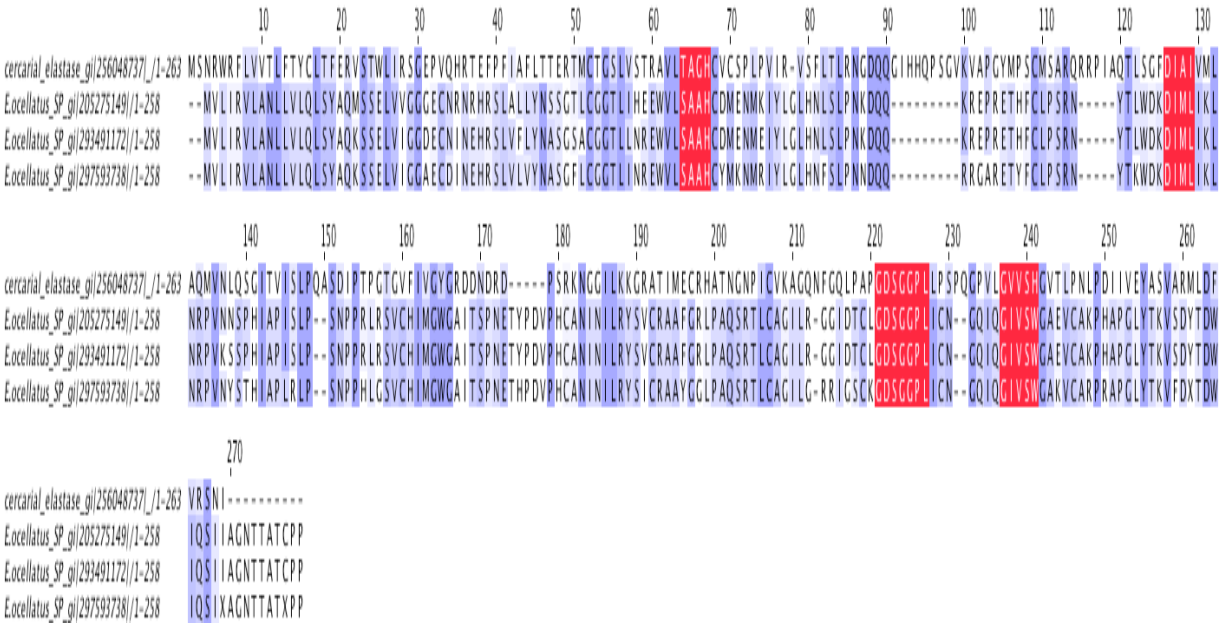
To initiate our investigation on the shared molecules between parasites, proteases and snake venom and to detect similarities in sequences representing these proteases, we carefully searched the literature for the types of proteases present in

parasites and their functions. We were mainly considering parasites proteases that are belong to the same protein family that major snake venom toxin proteases belong to. Several proteases found to be vital for the initiation of parasitism and the sustainability of parasite infection were identified. Of these, serine proteinases like; cercarial elastase (Newport et al. 1988; Salter et al. 2002), SmSP1, a kallikrein-like serine peptidase (Rawlings et al. 2008; Cocude et al. 1997) and SmDPP IV (Curwen et al. 2006). Metalloprotinases like; leucyl aminopeptidase (LAP) (Burley et al. 1990) and dipeptidyl peptidase III (DPP III) (Verjovski-Almeida et al. 2003) have been extensively characterized. Other trematode proteases have been annotated in EST databases, for example, cysteine peptidases (Robinson et al. 2008) and aspartic peptidases (Wong et al. 1997).

Next, to determine whether there were regions of similarity that may be a consequence of functional relationships between protein sequences of trematode (*S. mansoni* and *F. hepatica*) proteases and snake venom proteases, the constructed databases populated from publicly available assembled contigs of schistosome proteases and the available fasciola adult-stage data were blasted against the protease data of snake venom (*Echis ocellatus*) (Wagstaff and Harrison 2006). The present dataset was subjected to blastp analysis using an Intel, dual 2.8GHz RedHat Enterprise Linux 9.0 workstation running Biolinux 6.0 (available at http://envgen.nox.ac.uk) to identify putative orthologous between trematodes (*S. mansoni* and *F. hepatica*) and snake venom proteases.

Several schistosome proteases were showed to be orthologous to major toxic proteases sequences in snake venom (Figures 3.2-3.3). Interestingly, cercarial elastase—the serine protease critical for the initiation of schistosomiasis infection (Salter et al. 2000)—bore sequence similarities to snake venom serine protease using permissive (E-value $<1E^{-05}$). Cercarial elastase is a clan SA trypsin family serine protease able to cleave insoluble elastin, a major component of the dermis of skin (Salter et al. 2002). Although the maximum identity per cent between parasite protease and venom protease was found to be 25%, which is low in term of homology, however, it contains local regions that represent homology. Moreover, these homologues regions contain two motifs that are known to surround serine proteinase evolutionary markers and active site residues for the S1 family of clan

SA, TAAHC around His57, DIAL around Asp 102, GDSGGP around Ser195 and GIVSW for Ser214 (the residues' numbers are according to chymotrypsinogen numbering) (Krem and Di Cera 2001) (Figure 3.2).

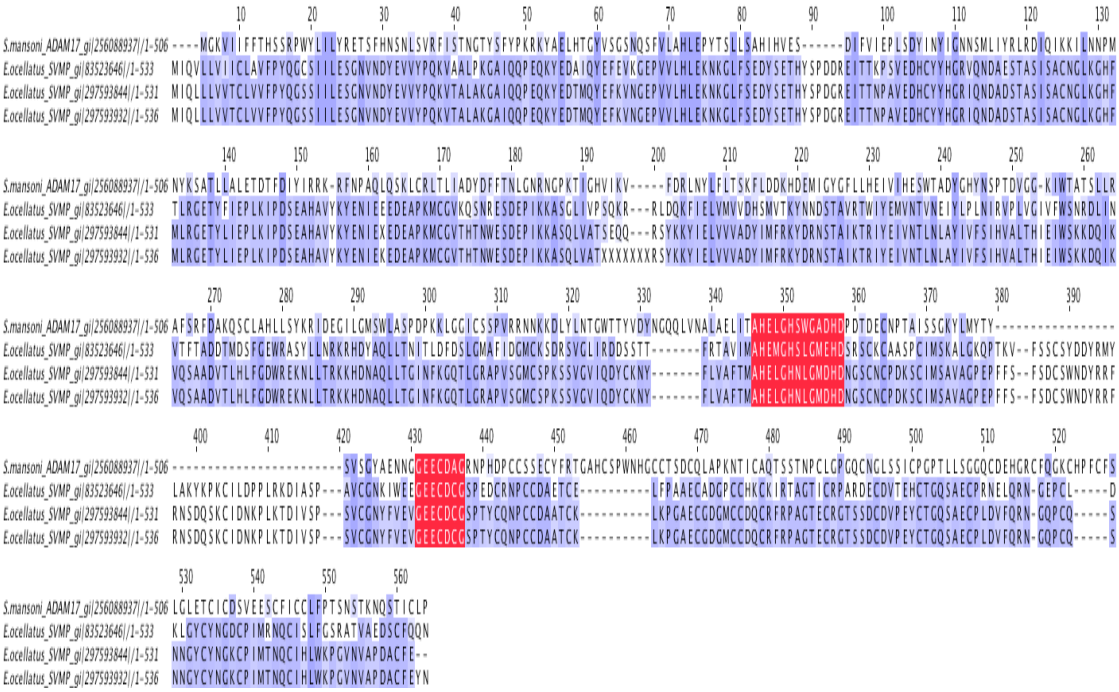


Description	Max score	Max score	Query cover	E value	Max ident	Accession
gi|205275149| serine protease *Echis ocellatus*	57.8	57.8	97%	2e-14	25%	CAQ72889.1
gi| 293491172 | serine protease *Echis ocellatus*	53.1	53.1	97%	5e-10	25%	ADE45140.1
gi| 297593738 | serine protease *Echis ocellatus*	52.4	52.4	96%	8e-10	24%	ADI47552.1

Figure 0.2 Multiple sequence alignment of *S. mansoni* cercarial elastase with *Echis ocellatus* serine protease isoforms. Comparative CLUSTAL (Jalview) sequence alignment of cercarial elastase (gi|256048737| with *Echis ocellatus* (gi|205275149|), (gi|293491172|) and (gi| 297593738 |) serine protease. Identities are higlighted in blue. Red shades highlight motifs known to surround serine proteinase evolutionary markers and active site residues for the S1 family of clan SA.

Similarly, metalloprotease from parasites, specifically A Disintegrin And Metalloproteinase 17 (ADAM17) peptidase, displayed similarities with group III

snake venom metalloproteinase (SVMPs). The maximum identity per cent between the two proteins was 53% (Figure 3.3). Several motifs showed conservation between the two proteins; AHEMxGHSxGxxHD, the two-histidine motif, which embedded in an active-site α-helix. In SVMP this motif lay within the metalloproteinase domain (Takeda et al. 2012). In this motif the glutamic acid or glu (E) is the catalytic residue (Gomis-Rüth 2003). In addition, another motif the GEECDxG that lay within the disintegrin domain in SVMP showed to be conserved between parasites and snake venom. The GEECDxG exhibited conservation between SVMPs and have high potential immunogenicity (Wagstaff et al. 2006). In general, SVMPs are grouped with the ADAMs protein family (Casewell 2012). However, ADAMs contain domains that are absent in SVMPs and they are widely recognised as highly diverse proteins with functional roles involving the shedding of membrane-bound protein domains and acting as molecular switches that activate other proteins by proteolysis (White 2003; Casewell 2012).



Description	Max score	Max score	Query cover	E value	Max ident	Accession
gi|83523646| Group III snake venom metalloproteinase *Echis ocellatus*	62.8	132	73%	2e-14	53%	CAJ01689.1
gi|297593844| Snake venom metalloproteinase *Echis ocellatus*	56.6	56.6	22%	4e-10	30%	ADI47605.1
gi|297593932| Snake venom metalloproteinase *Echis ocellatus*	56.2	56.2	22%	5e-10	30%	ADI47649.1

Figure 0.3 Multiple sequence alignment of *S. mansoni,* ADAM17 peptidase, with *Echis ocellatus,* Group III snake venom, metalloproteinase. Comparative CLUSTAL (Jalview) sequence alignment of schistosome ADAM17 peptidase (gi|256088937| with *Echis ocellatus* (gi|83523646|) Group III snake venom metalloproteinase, (gi|297593844|) and (gi|297593932|) snake venom metalloproteinase. Identities are highlighted in blue. Red shades highlight conserved motifs within the metalloproteinase domain of the protein.

1.8.2 Blast2GO Gene Ontology (GO) terms prediction

To gain a basic understanding of the protein functions in which they are involved and to better understand parallel functions between parasites and snake venom, molecular function GO terms were assigned to the constructed databases. Gene ontology provides structured, controlled vocabularies and classifications

covering different domains of molecular and cellular biology (Harris et al. 2004). The GO of parasites' peptidases was compared to the ontology of snake venom (*E. ocellatus*) peptidases. A total of 15,240 *S. mansoni* assembled EST contigs (average sequences length of 666 ± 194 bp) and 737 *F. hepatica* ESTs (average sequences length of 572± 143 bp) were subjected to GO annotation. The details of parameters used in this investigation are demonstrated in Figure 3.1. The successive annotation of the inferred proteins revealed a total of 3446 molecular function GO terms for the six different life stages of *S. mansoni* and 345 for the *F. hepatica* adult worm; details of each life stage are shown in Table 1. The predominant terms for the *S. mansoni* life cycle stages were binding (GO: 0005488), followed by catalytic activity (GO: 0003824) (Table 2). Similarly, binding (GO: 0005488) was the most abundant term for the *F. hepatica* adult worm, followed by protein binding (GO: 0005515) (Table 5).

Life stage	Number of assembled contigs	Average sequence length ± standard deviation	Returning IP results	Gene Ontology (molecular function)
Cercaria	3967	438 ± 6.44	358	590
Day 7 schistosomula	3101	723 ± 246.72	951	953
Lung-stage schistosomula	1364	633 ± 9.22	411	55
21-day adult	371	904 ± 164.60	113	346
28-day adult	450	845 ± 220.40	332	384
Egg	5987	454 ± 8.23	608	1118
Fasciola adult	737	572 ± 143	-	354

Table 0.1 Summary of the assembled contig data for the different life stages of *S. mansoni* and *F. hepatica* following a detailed bioinformatics annotation analysis.

From 3967 contigs of *S.mansoni* infective stage, the cercaria, that initiate the infection by penetration of host skin, a total of 590 molecular function GO terms were inferred. For proteases, metallopeptidase activity (GO: 0008237) was the predominant term with 0.18%, followed by cysteine-type peptidase activity (GO: 0008234) with 0.10%. The frequency of serine-type peptidase activity (GO: 0008236) was 0.08% and 0.03% for threonine-type peptidase activity (GO: 0070003); however, no function was inferred for aspartic-type peptidase activity (GO: 0070001) (Table 3.2). A total of 358 proteins were predicted from cercaria contigs in InterProScan; 162 proteins were predicted as domain, 62 as conserved site and 93 as family, as well as others such as active site and binding site (Table 3.3).

In 7-day schistosomula, which travel through skin layers to enter the host's circulation from the dermal lymphatics and venules, 3101 assembled contigs were bioinformatically studied. A total of 953 GO molecular function terms were detected. However, unlike cercaria, all five types of proteases were identified in 7-day schistosomula. Cysteine-type peptidases (GO: 0008234) were first, with 0.48%, followed by 0.26% for metallopeptidase activity (GO: 0008237) (Table 3.2).

Of the total identified molecular function GO terms, aspartic-type peptidase activity (GO: 0070001) was highest with 0.16%, followed by serine-type peptidase activity (GO: 0008236) with 0.13% and threonine-type peptidase activity (GO: 0070003) with 0.10% (Table 3.2). An InterProScan study revealed 951 different proteins, 416 of which were domain, 308 family and 96 conserved sites (Table 3.3).

GO code	GO term	Cercaria %	Day 7 schistosomula %	Lung-stage schistosomula %	Day 21 adult %	Day 28 adult %	Egg %
GO:0003674	Molecular function	13.03	28.31	35.92	33.96	37.78	22.10
GO:0005488	Binding	11.19	24.96	32.04	31.54	34.22	19.86
GO:0003824	Catalytic activity	8.24	15.09	18.99	16.71	16.67	11.63
GO:0016787	Hydrolase activity	5.14	7.32	10.12	7.55	7.33	6.10
GO:0008233	Peptidase activity	0.45	1.32	1.91	1.89	1.11	0.68
GO:0004175	Endopeptidase activity	0.33	1.06	-	1.08	0.44	0.45
GO:0008238	Exopeptidase activity	0.05	0.10	-	0.27	0.22	0.13
GO:0070001	Aspartic-type peptidase activity	-	0.16	-	0.27	-	-
GO:0004190	Aspartic-type endopeptidase activity	-	0.16	-	0.27	-	-
GO:0008234	Cysteine-type peptidase activity	0.10	0.48	-	0.27	0.44	0.13
GO:0004197	Cysteine-type endopeptidase activity	0.08	0.39	-	0.27	0.44	0.08
GO:0008237	Metallopeptidase activity	0.18	0.26	-	0.27	0.22	0.17
GO:0004222	Metalloendopeptidase activity	0.13	0.16	-	0.27		0.05
GO:0008235	Metalloexopeptidase activity	-	0.06	-	-	-	0.13
GO:0004181	Metallocarboxypeptidase activity	-	-	-	-	-	0.05
GO:0008236	Serine-type peptidase activity	0.08	0.13	-	0.27	0.44	0.12
GO:0004252	Serine-type endopeptidase activity	0.08	0.06	-	-	-	0.12
GO:0070003	Threonine-type peptidase activity	0.03	0.10	-	0.27	-	0.08
GO:0004298	Threonine-type endopeptidase activity	0.03	0.10	-	0.27	-	0.08
GO:0070011	Peptidase activity, acting on L-amino acid peptides	0.43	1.29	-	1.62	1.11	0.65
GO:0004177	Amino-peptidase activity	-	0.03	-	-	-	0.08
GO:0016805	Dipeptidase activity	-	0.03	-	-		0.02
GO:0004180	Carboxypeptidase activity	0.03	-	-	-	0.22	0.05
GO:0004623	Phospholipase A_2 activity	0.05	0.03	-	0.27	-	0.03

Table 0.2 The three most abundant GO terms and a detailed description of the protease family's GO terms. The percentage indicates the number of sequences with the described GO term out of the total number of sequences uploaded to the software.

Only 55 molecular function GO terms from 1364 assembled contigs of SLAP were characterised (Table 3.2). Although the parent term peptidase activity was inferred from the GO term analysis with 1.91%, not one of the five types of proteases appeared in the results. In the InterProScan study, 411 proteins were detected, with 144 domain, 153 family and 63 conserved sites.

Similarly, in the 21-day adult worm, which resides in the hepatic portal system, 346 molecular function GO terms were inferred from 371 assembled contigs. All five peptidase types (cysteine-type peptidases (GO: 0008234), metallopeptidase activity (GO: 0008237), aspartic-type peptidase activity (GO: 0070001), serine-type peptidase activity (GO: 0008236) and threonine-type peptidase activity (GO: 00703)) occurred with the same percentage of 0.27% (Table 3.2). The InterProScan results showed 113 proteins differentiated in 73 domains, 21 families and 8 conserved sites as well as 9 PTM and 2 repeats (Table 3.3).

InterProScan domain	Cercaria	Day 7 schistosomula	Lung-stage schistosomula	21-day adult	28-day adult	Egg
Domain	163	417	145	73	115	257
Family	94	309	154	21	155	146
Conserved site	69	97	64	8	38	113
Binding site	2	20	2			17
Active site	6	34	8		12	29
Region	13	38	30	9	10	22
PTM	7	4				13
Repeat	4	32	8	2	2	11

Table 0.3 Protein domains inferred using the InterProScan analysis from B2GO software from peptides inferred from schistosoma life cycle stages.

In the 28-day adult, aspartic-type peptidases activity (GO: 0070001) and threonine-type peptidase activity (GO: 0070003) were not detected in the 384 GO terms retrieved from the 450 assembled contigs. Cysteine-type peptidases (GO: 0008234) and serine-type peptidase activity (GO: 0008236) were similar at 0.44%, while the metallopeptidase activity (GO: 0008237) percentage was 0.22% (Table

3.2). InterProScan analysis revealed 332 proteins; 155 were families and 115 were domains (Table 3.3).

Finally, the egg stage that is laid intravascularly and many are carried away from the site of deposition by blood flow, to become lodged in the nearest capillary bed down-stream or excreted with faeces. 5987 assembled contigs were subjected to Blast2GO GO analysis, which resulted in 1118 molecular function GO terms. Metallopeptidase activity (GO: 0008237) was the most abundant protease result with 0.17%. Cysteine-type peptidases (GO: 0008234) came next with 0.13%, followed by 0.12% for serine-type peptidase activity (GO: 0008236) and finally 0.8% for threonine-type peptidase activity (GO: 0070003). No results were detected for aspartic-type peptidase activity (GO: 0070001) (Table 3.2). After screening the assembled contigs using InterProScan, 608 proteins were detected—257 were domains, 146 were families and 113 were conserved sites. Others such as binding sites, active site regions and repeats were identified as well (Table 3.3).

This analysis demonstrates that GO categories with an interesting enrichment of Schistosome life stages over all schistosome proteins are “binding function” (including calcium- and lipid binding proteins) and “enzymatic activity” (including the hydrolytic and catalytic enzymes). Proteases of all classes are expressed in different stages of the parasite. In addition, we can notice the differences among different proteases expressions at the different stages, especially those that consider being consecutive, like cercaria and schistosomula. This may be related to the tissue compartment or body part the parasite resides in and the different adaptations it undergoes to ensure the success of parasitism.

F. hepatica adult worm inferred a total of 354 molecular function GO terms, of which binding (GO: 0005488) was the predominant one, with 22.7% of the total number of loaded sequences. Protein binding (GO: 0005515) was the second with 20.7%. Peptidase activity was inferred from 8.0% of the loaded ESTs (Tables 3.1, 3.4). Notably, peptidases of the cysteine and serine types were the only types of peptidases detected by this analysis. Unlike schistosomes, there were no inferred

catalytic or hydrolytic GO terms. In addition, from the proteases point of view, no metalloproteases, aspartic proteases or threonine proteases were detected, although the percentage of the GO term-detected peptidases was higher in fasciola than in the schistosome lifecycle stages. The InterProScan result was negative, which could be because the fasciola database was released recently and has not yet been annotated or analysed in detail.

GO Code	GO term	*F. hepatica* adult worm
GO:0003674	Molecular_function	24.0%
GO:0005488	Binding	22.7%
GO:0005515	Protein binding	20.6%
GO:0070011	Peptidase activity, acting on L-amino acid peptides	8.0%
GO:0008233	Peptidase activity	8.0%
GO:0004175	Endopeptidase activity	7.7%
GO:0004197	Cysteine-type endopeptidase activity	7.2%
GO:0008234	Cysteine-type peptidase activity	7.2%
GO:0008236	Serine-type peptidase activity	0.8%
GO:0004252	Serine-type endopeptidase activity	0.5%
GO:0008238	Exopeptidase activity	0.3%
GO:0004869	Cysteine-type endopeptidase inhibitor activity	0.3%
GO:0008239	Dipeptidyl-peptidase activity	0.3%

Table 0.4 The three most abundant GO terms and a detailed description of the protease family GO terms. The percentage indicates the number of sequences with the described GO term out of the total number of sequences uploaded to the software.

For comparative analyses, *E. ocellatus* EST data generated at the Alistair Reid Venom Research Unit was also included. For this dataset, the same bioinformatic analyses described in the Methods section were conducted. From 113 contigs, a total of 101 molecular function GO terms were inferred, with most of them matching those encoded by schistosome and fasciola. However, several inferred molecular function GO terms from *E. ocellatus* were not detected in parasites—

peptidyl-dipeptidase inhibitor activity (GO:0060422), L-amino-acid oxidase activity (GO:0001716), cytokine binding (GO:0019955), metalloenzyme regulator activity (GO:0010576), hyaluronon glucosaminidase activity (GO:0004415) and prostaglandin-endoperoxide synthase activity (GO:0004666). It is noteworthy that all of the snake venom proteases were under endopeptidase molecular function, whereas the parasite proteases were divided between endo- and exopeptidases.

		Schistosoma mansoni							
GO code	**GO term**	**Cercaria %**	**Schistosomula %**		**Day 21 adult %**	**Day 28 adult %**	**Egg %**	**Fasciola hepatica adult %**	**Echis ocellatus %**
			Day 7	**Lung stage**					
Go:0003674	Molecular function	13.03	28.31	35.92	33.96	37.78	22.1	24	77.1
Go:0005488	Binding	11.19	24.96	32.04	31.54	34.22	19.86	22.7	58
Go:0003824	Catalytic activity	8.24	15.09	18.99	16.71	16.67	11.63		75.6
Go:0016787	Hydrolase activity	5.14	7.32	10.12	7.55	7.33	6.1	0	51.1
Go:0008233	Peptidase activity	0.45	1.32	1.91	1.89	1.11	0.68	8	47.3
Go:0004175	Endopeptidase activity	0.33	1.06	0	1.08	0.44	0.45	7.7	47.3
Go:0008238	Exopeptidase activity	0.05	0.1	0	0.27	0.22	0.13	0.3	0
Go:0070001	Aspartic-type peptidase activity	0	0.16	0	0.27	0	0	0	1.5
Go:0008234	Cysteine-type peptidase activity	0.1	0.48	0	0.27	0.44	0.13	7.2	0
Go:0008237	Metallopeptidase activity	0.18	0.26	0	0.27	0.22	0.17	0	38.2
Go:0008236	Serine-type peptidase activity	0.08	0.13	0	0.27	0.44	0.12	0.8	7.6
Go:0070003	Threonine-type peptidase activity	0.03	0.1	0	0.27	0	0.08	0	0

Table 0.5 Comparison of protease molecular function GO terms between parasites (*S. mansoni* and *F. hepatica*) and snake venom (*E. ocellatus*) proteases.

1.9 Discussion

In this chapter, the *in silico* approach was employed to elucidate fundamental molecules that are critical to success of parasitism/envenomation and share parallel nature in parasites and snake venom. The transcriptome of these pathogens' data was bioinformatically investigated and compared. Comparative analyses between transcriptome of different organisms using GO comparison have been used to gain understanding of molecular and biological aspects (Cantacessi et al. 2010; Conesa & Götz 2008). The assembled contig library of each life stage of *S. mansoni* (cercaria, 7-day schistosomula, SLAP, 21- and 28-day adult and soluble egg antigen) and adult *F. hepatica* involved migration or caused pathological damage to the host, was surveyed for orthologous in protein sequences and molecular function GOs comparison with snake venom database. These parasites stages use different proteins and proteases, like cercarial elastase, metalloprotease and aspartic protease, to facilitate the destruction of tissue compartments laying the parasite way to the final habitat, digest blood meal obtained from host or evade the immune system of the host. In comparison, snake venom use similar proteases to destroy systemic integrity of envenomed patient and cause profound coagulopathy and myotoxicity as well as neurotoxicity.

The BLASTp and alignment of *S. mansoni* and *F. hepatica* protease ESTs against snake venom ESTs revealed orthologues among essential enzymes in the pathology of schistosoma and fasciola and major toxins in snake venom. The serine protease cercarial elastase (chymotrypsine family) showed 25% identity with snake venom serine protease (SVSP) (accession numbers CAQ72889.1 and ADI47555.1), as shown in Figure 3.1 in the Results section. Although this identity is considered to be low, it includes serine proteinase evolutionary markers and active site residues for the S1 family of clan SA. Further, TAAHC, DIAL, GDSGGP and GIVSW with their development and maintenance played an important role in the evolution of these proteolytic enzymes as variation in active site structure permit the enzyme to fulfill new roles (Krem & Di Cera 2001). The homology between serine proteases from the two different organisms was represented in other serine protease isoforms from schistosome that had an identity of 32% with SVSP (accession numbers ADI47559.1 and ADI47560.1). Similar to cercarial elastase, SmSP1, another serine protease

found in schistosomes, shared motifs with snake serine proteases that surround the active sites of clan A S1 serine protease family similar to that showed in cercarial elastase.

Functionally, cercarial elastase is a predominant secreted protease that is essential for host skin invasion due to its ability to digest insoluble elastin (Ingram et al. 2012). Furthermore, an isoform to cercarial elastase localised on the surface of schistosomula has been implicated in immunomodulatory roles, including the cleavage of host complement components and of host IgG and IgE bound via Fc receptors to their surface (Tort et al. 1999; Kasný et al. 2009).

On the other hand, SmSP1 of adults was shown to cleave bradykinin and provoke a reduction of the arterial blood pressure of experimental animals (rats), possibly due to a peripheral vasodilatation effect. Thus, parasite enzymes might have a similar influence on the visceral vasculature and capillary permeability of their natural hosts (Carvalho et al. 1998; Kasný et al. 2009). In comparison, SVSPs affect the pathways of the coagulation cascade by activating blood components utilised in the coagulation cascade, fibrinolysis and platelet aggregation or by proteolytic degradation (Serrano and Maroun 2005). This is in addition to characterised SVSPs that are able to lower the blood pressure and bring about disturbances of the hemostatic function (Serrano et al. 1998). This correlates SVSPs with SmSP1 more than cercarial elastase as SmSP1 is known, as described above, to induce the reduction of blood pressure.

Other important proteases such as metalloprotease shared some identity (53%, accession number CAJ01689.1) with snake venom metalloprotease (SVMP) (Figure 3.2). In human active schistosomal periovular granulomas, metalloproteinases are key enzymes in the metabolism of collagen and in the physiopathology of fibrosis (Gomez et al. 1999). Similarly, in snake envenomation, SVMPs are responsible for haemorrhage and multiple tissue-damaging activities, including myonecrosis, dermonecrosis, blistering and oedema (Takeda et al. 2012; Watanabe et al. 2003).The schistosome metalloprotease belongs to the ADAM family of metalloprotease. Snake venom metalloprotease found to be closely related to the ADAM family of metalloproteases (Takeda et al. 2012) this may explain the

expressed identity between schistosome metalloproteases and SVMP.

In GO analysis performed using B2GO, comparing the inferred terms of *S. mansoni* and *F. hepatica* with the snake venom protease database indicated shared molecular functions in their proteases, which were represented in the organisms (Table 3.5). These proteases included cysteine, aspartic and metalloproteases, which are known to function in multi-enzyme cascades to perform different biological functions. Several observations were made. All of the snake venom proteases inferred in our study were under endopeptidase molecular function, whereas the parasite proteases were divided between endo- and exopeptidases. In this regard, snake venom proteins are synthesised in secretory epithelial cells lining the tubules of the venom gland (Mackessy & Baxter 2006). The most commonly quantified classes of snake venom enzymes include phospholipase A_2 (PLA_2), phosphodiesterase, phosphomonoesterase, L-amino acid oxidase, specific endopeptidases and nonspecific endopeptidases (McCue 2005). More than 150 different endoproteases have been isolated and about one third of these have been structurally characterised (Ogawa et al. 2007; Vaiyapuri et al. 2010). In contrast, little information is available about the functions of exopeptidases (aminopeptidases and dipeptidyl peptidases), which are capable of hydrolysing the N-terminal residue(s) of peptides in venom (Ogawa et al. 2007). Several studies demonstrated the presence of aminopeptidase in snake venom, for example, Aminopeptidase A (APA), Aminopeptidase B (APB), Aminopeptidase N (APN) and Dipeptidyl peptidase (DPP); however, the roles of these aminopeptidases in snake venom have not yet been fully understood (Ogawa et al. 2007; Vaiyapuri et al. 2010; Aird et al. 2013). This may in a way explain why parent GO terms related to exopeptidases were not inferred in our analysis for *E. ocellatus* venom, and metalloenzyme inhibitor activity (GO:0048551) and metalloenzyme regulator activity (GO:0010576) were the only GO terms related to exopeptidases inferred through this analysis.

On the other hand, exopeptidases have been closely studied in Schistosome. Exopeptidases in Schistosomes likely play a role in haemoglobin digestion following endopeptidases activity (Merrick et al. 2003). Several schistosome exopeptidases have been reported, including cathepsin C, cathepsin B, aminopeptidase and dipeptidyl peptidase (Sajid 2003; Kasný et al. 2009; Rawlings et al. 2008; Myers et al. 2008).

When comparing the different life stages of *S. mansoni*, the variations in the percentage of proteases at each stage inferred in our study were obvious. This might reflect the different biological functions of the parasite at each stage according to the nature of the site of that stage inside the host. This starts with cercaria, which initiates the infection by penetrating the host skin using acetabular gland contents in which cercarial elastase (serine protease) plays a vital role (El-Ansary 2003; Bahgat et al. 2002). At this stage, the parasite needs to stimulate blood clotting, which can seal ruptured capillaries during parasite migration, in which serine protease and metalloprotease may participate (Dvorák et al. 2008; Ingram et al. 2003). This demonstrate the presence of serine protease and metalloprotease GO term in the inferred molecular functions (Table 3.2) Obviously, no aspartic protease GO term was inferred from this stage, which might correlate with the fact that the parasite at this stage is not digesting blood from the host. In schistosomula, the consecutive stage, we notice an increase in the level of proteases GO terms as this stage actively migrates through tissue compartments, may reach the lung alveoli and start to contact host blood. Hence, it may start feeding on blood and digestion facilitated by aspartic proteases (Miller and Wilson 1978; Harrop et al. 1999). On the other hand, the absence of some of these fundamental proteases at some stages of *S. mansoni* may be related to the yet incomplete sequencing project (Table 3.2). The adult stage at which the parasite has reached the final habitat secretes proteases for the maintenance and sustainability of the infection (El-Ansary 2003; Curwen et al. 2004; Guerra-Sá et al. 2005). This stage utilises proteases for host blood digestion and the escape and evasion of the host's immune system. Proteases like cercarial elastase, cathepsin B (Sm31), cathepsin L1, cathepsin L2, cathepsin D, cathepsin C and legumain (Sm32) are capable of modulating the host immune response and regulating the synthesis of specific and other IgE antibodies (El-Ansary 2003). This is reflected in our finding as all type of proteases GO terms was detected. On the other hand, eggs are deposited by the adult stage and are trapped in the tissues of the infected host, inducing granuloma, portal fibrosis, hepatosplenomegaly and gastrointestinal varices (Pearce and MacDonald 2002; Rinaldi et al. 2009). Proteases have also been found to play a role in this process (Ashton et al. 2001; Cesari et al. 2000; Morales et al. 2008). Similar to Schistosome, *F. hepatica* utilises different

proteases during the route of the infection in order to ensure the success of parasitism (Stack et al. 2011; Boukli et al. 2011; Kasný et al. 2009).

The preliminary data presented in this chapter provides a basic overview of the presence of shared functions between proteases utilized by migrating parasites and snake venom as both use proteases to facilitate and sustain pathology. It is interesting to investigate further if these trascriptomics similarities will extend to represent *in vitro* reactions on the proteomic level. Wither serine proteases, metalloproteases and other proteases used by parasites during tissue migration, immune system evasion, host blood digestion, vasodilatation and granuloma formation will be detected with antivenoms and antibodies raised against venom full of proteases utilised by snakes to deliver venom to the victim's blood system. This venom's components disturb the victim's natural systems, affecting haemostasis and vasodilatation, therefore causing damage to local tissue and systematic pathology. This will be investigated in the next chapter.

Chapter 4 Immunological analysis of the cross-reactivity of parasite and venom proteins

1.10 Introduction

Molecules shared among species of various genera, families, and phyla have been widely described. The expression of immune responses among different species of various genera, known as antigenic community, is responsible for antigenic cross-reactivity (Boukli et al. 2011; Losada et al. 2005). Sharing molecules among different organisms is an expected result of the conserved function of many molecules, such as enzymes, hormones, and receptors, during evolution (Boukli et al. 2011). This characteristic has special relevance for identifying molecules with potential for the development of drugs or vaccines that could be effective against different species or genera of organisms.

In the previous chapter, we demonstrated the presence of similar enzymes used by the *S. mansoni* and *F. hepatica* parasites and snake venom to trigger pathology. For instance, cercarial elastase, which is the major larval protease in *S. mansoni* and essential for host skin invasion (Ingram et al. 2012; Knudsen et al. 2005) has a shared molecular function with serine proteases from snake venom and displays a degree of sequence homology with venom serine proteases. In addition to cercarial elastase, both parasites and venom use other enzymes to ensure success and sustainability for infection/pathology, such as phospholipases, aspartic proteases, metalloproteases and hyaluronidase, which is a component found in snake venom and parasites, that is able to spread degradation of extracellular matrix that may contribute to the spreading of venom toxins (Gutiérrez et al. 2007; Levine et al. 1948; Evans 1953).

However, the literature has stated that the transcriptome of an organism does not necessarily correlate with the translatome and protein expression in biological samples (Maier et al. 2009). Post-transcriptional regulation of synthesis and the degradation rate of a protein have an impact on molecule differentiation, and thus, affect the correlation between transcriptome and proteome (Kristensen et al. 2013). Therefore, in this chapter, we aimed to further investigate this similarity on the protein level, using immunological and proteomic approaches. Knowing that

antivenom has the ability to bind to most venom toxin proteins, because horses/sheep are hyperimmunised with venom, we aimed to identify the parasite proteins bound by antivenom.

Taking into account the proteases we are interested in, we selected a group of antivenom- and toxin-specific immunoglobulin Gs (IgGs) for the investigation. We compared parasite protein cross-reactivity to i) the venoms of snakes that are life threatening and a daily hazard in large tracts of Africa, and which share an epidemiological distribution with schistosome and fasciola parasites. Thus, *E. ocellatus* venom was selected as our element of comparison with parasites in our experiments. Serine proteases and metalloproteases are major components of this snake venom, and they are thought to disrupt several distinct elements of vascular haemostasis of envenomed victims (Wagstaff & Harrison 2006). As such, it is a good example for our comparison, and ii) a monospecific antivenom, EchiTAB G, which neutralizes the toxic effect of *E. ocellatus* venom (Abubakar et al. 2010), was selected as the primary antibody for our experiments

To increase our chances of identifying additional antigenically cross-reactivevenom and parasite proteins, we broadened our antivenom selection to polyspecific antivenoms produced against a mix of vipers and elapids, because of the precence of a wider spectrum of IgG specificities to these elapid venom proteins (e.g. neurotoxins). Thus, we included the African polyspecific antivenoms EchiTAb-PLUS-ICP and SAIMR polyvalent (Abubakar et al. 2010; Mebs et al. 1988). We included in this analysis polyspecific antivenom developed against non-African snake venom as a control and to investigate whether the shared molecules exist in snakes found in areas that do not encounter schistosomiasis. Mass-spectrometry analysis of these protein was performed to elucidate which parasite proteins immunoreacted to the antivenoms and to link the reactive protein of the antibodies to what we observed using transcriptome data.

Finally, to exclude non-specific binding to other antivenom components, an immunoblot was performed with antibodies raised to specific *E. ocellatus* venom protein groups.

1.11 Materials

The source and preparation of the samples are described in detail in Chapter 2, Section 2.1.

1.12 Methods

1.12.1 Electrophoresis analysis and immunoblotting

Sample homogenates were size-separated by SDS-PAGE (15%), then electroblotted to 0.45 μm nitrocellulose membranes, according to the manufacturer's (Bio-Rad, UK) recommendations, as described in Chapter 2, Section 2.6. The membranes were incubated overnight in a variety of polyclonal and monoclonal snake antivenoms and toxin-specific IgGs (Table 4.1), at 1:5000 and 1:200 dilutions respectively, in a blocking buffer.

We began the experiments using the *E. ocellatus* monospecific antivenom (EchiTAb G®) which has the ability to neutralize the most clinically relevant toxic activities (lethal, haemorrhagic, coagulant, and necrotizing) of this venom (I. S. Abubakar et al. 2010). Selection of this antivenom was based on our rationale of the presence of similar proteins between parasites that reside in the capillaries of a human host and have the ability to disturb tissue compartment integrity and migrate to the vascular system, and venoms of snakes that cause tissue necrosis and interrupt haemostasis in their victims. Our interest in African snakes was motivated not only by the shared biological mechanisms between these different organisms, but also, as described in Chapter 1, by the fact that these pathological organisms exert health issues on populations that reside in the same geographical locations. To upgrade this investigation, we broadened our antivenom specificity to the polyspecific EchiTAb-Plus-ICP (S. B. Abubakar et al. 2010) and SAIMR antivenoms (Mebs et al. 1988), which are effective against venoms of not only *E. ocellatus*, but also other toxic snakes, such as vipers and elapids, which cause different pathologies (e.g. elapids cause neurotoxic pathology) in Africa (Table 4.1). The selection of these

polyspecific antivenoms was to cover more medically important snakes and to investigate whether broadening venom selection would increase the possibility of detecting more shared proteins. Finally, to test whether this cross-reactivity is to African snakes only or if it is true of other snakes distributed in non-African countries, we used antivenom (Polyvalent Snake Antivenom; CSL Australia) developed against snakes found in Australia and nearby countries (O'Leary and Isbister 2009). We considered the fact that the source of antibodies raised against venom might affect the background of the immunoblots, as sheep, for example, might have been infected with parasites that share biological molecules with parasites we used in our experiment. Therefore, we tried to select antivenom raised in equines as well as in ovines.

Snake Antivenom	Snake	IgG type	Reference
EchiTAb G®; carpet viper monospecific (MicroPharm, UK)	*E. ocellatus*	Ovine	(Abubakar et al. 2010 (a); Abubakar et al. 2010 (b))
EchiTAb-Plus-ICP, African-polyspecific (Instituto Clodomiro, University of Costa Rica)	*E. ocellatus, B. arietans, N. nigricollis*	Equine	(Gutiérrez et al. 2005)
SAIMR Polyvalent, African-polyspecific (South African Institute for Medical Research (SAIMR), Johannesburg, South Africa)	*B. arietans*, *B. gabonica*, *N. annulifera, N. melanoleuca*, *N. mossambica, D. polylepis*, *D. angusticeps*, *D. jamesoni*, *H. haemochatus*	Equine	(Mebs et al. 1998)
Polyvalent Snake Antivenom (Australia–Papua New Guinea) Australian-polyspecific (CSL Limited, Australia)	*P. australis, N. scutatus,* *P. textilis, A. antarcticus, O. scutellatus*	Equine	(O'leary and Isbister 2009)

Table 0.1 Snake antivenom used in this study.

1.12.2 Mass spectrometry

The detailed description of this experiment is illustrated in Chapter 2 sections 2.7.a-b

1.13 Results

1.13.1 Protein profiling

To determine the protein composition of the parasite life cycle, homogenates were fractionated by 15% SDS-PAGE electrophoresis (Figure 4.1). The parasite protein profile showed a more complex protein composition compared with snake venom, which is logical, as the parasite samples, except cercarial transformation fluid and soluble egg antigen, were homogenates for the whole worm stage and not secretory/excretory fluids only. There were variations in the parasite protein profiles separated by reducing SDS-PAGE of specimens prepared from different schistosome stages. For instance, when comparing the cercaria (lane 1 in Figure 4.1) and schistosomula (lane 2 in Figure 4.1) stages, which are two consecutive stages of the parasite, we noticed a big difference in their profiles. This shows the complex morphological and physiological changes that occur in the individual life-stages of the parasite and displays the distinct adaptations of the parasite to both parasitic life and free living.

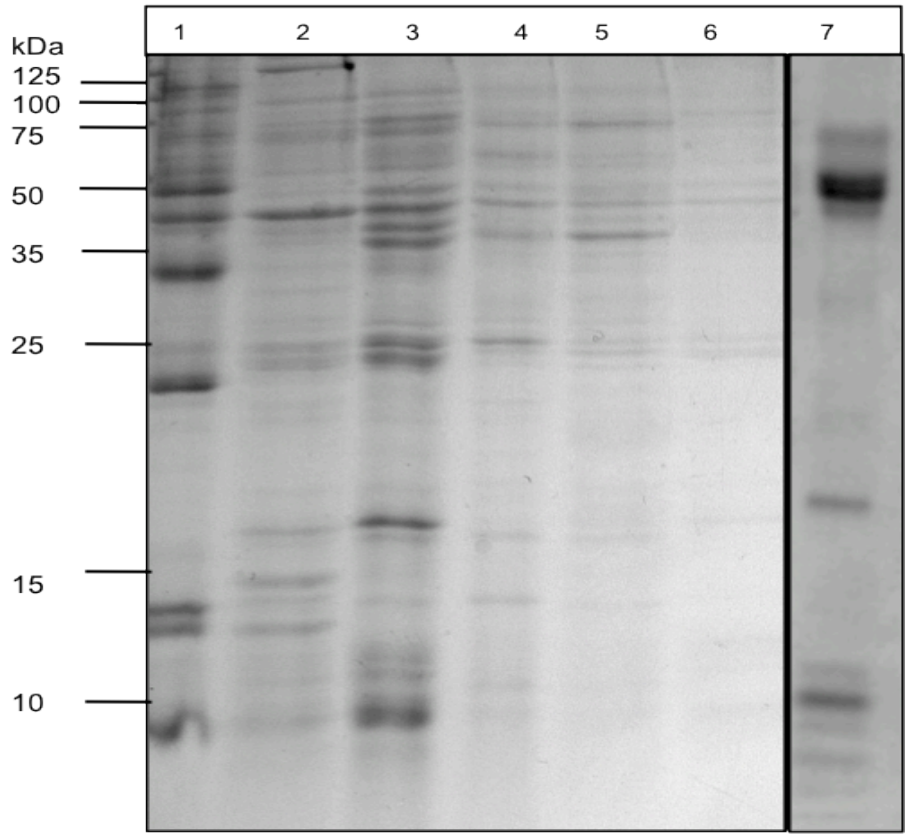


Figure 0.1 Analysis of parasite stages and venom proteins: 15% reduced SDS-PAGE gel stained with Coomassie blue of the following samples: *Schistosoma mansoni* life cycle stages (**1** cercaria, **2** cercarial transformation fluid, **3** schistosomula, **4** *S. mansoni* adult, **5** soluble egg antigen), **6** *Fasciola hepatica* adult worm proteins, and **7** *Echis ocellatus* venom proteins. Samples were normalized for loading according to the best protein amount to have the strongest reaction to antibodies used in the blots as in the following sections. The molecular weight markers (kDa) are shown on the left side of the image. Note the complexity of the protein composition of the parasite.

1.13.2 Protein immunoreactivity analysis

An immunoblotting assay was performed to identify the *S. mansoni* and *F. hepatica* proteins that reacted with a variety of mono- and poly-specific snake antivenoms (Table 4.1).

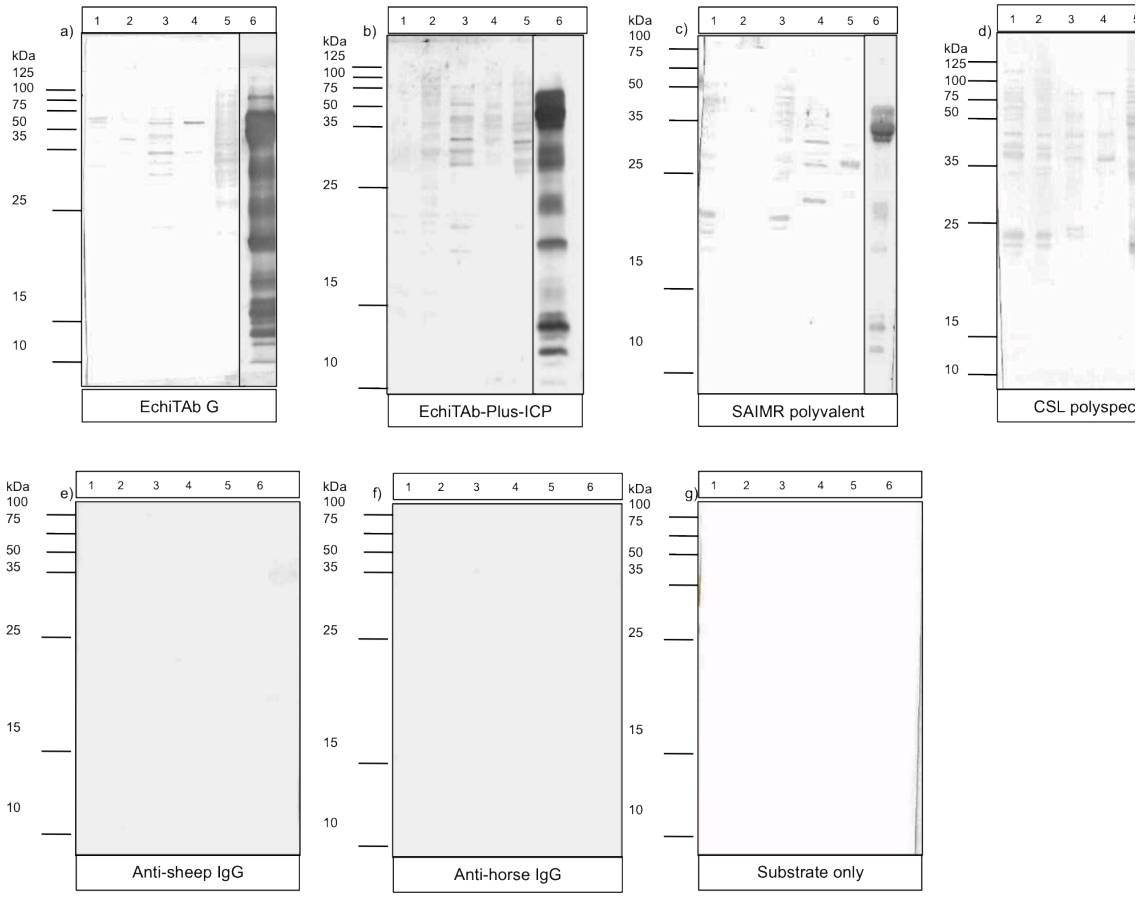


Figure 0.2 Reduced (15% SDS-PAGE gel) immunoblotting against various antivenoms: African a) monospecific EchiTAb G, b) polyspecific EchiTAb-Plus-ICP, and c) SAIMR polyvalent; and Australian d) CSL polyspecific. As controls: secondary antibody only e) donkey anti-sheep IgG, f) rabbit anti-horse IgG, and g) substrate only. 5µg of samples were loaded in the following order: 1) *S. mansoni* cercaria, 2) *S. mansoni,* schistosomula, 3) *S. mansoni* adult, 4) *S. mansoni* SEA, 5) *F. hepatica* adult, 6) *E. ocellatus* venom as positive control for the African antivenoms, *6) *Acanthophis antarcticus* as a positive control for the Australian antivenom CSL. The molecular weight markers (kDa) are shown on the left side of the image.

Monospecific EchiTABG

First of all, we used the monospecific EchiTAb G antivenom, raised against *E. ocellatus* venom, which is dominated by, Zn^{2+}-metalloproteinases, and group II phospholipases A_2 that disrupts. Immunoblotting analysis revealed differences in the reactivity of distinctt Schistosome life-cycle stages toward the antivenom. Starting with the *S. mansoni* infective stage, cercaria (lane 1, Figure 4.2a), which reacted with the MW 30kDa protein and three consecutive bands between MWs 50kDa and

55kDa, this was different from schistosomula, the stage following the cercaria, and the stage spent migrating through tissue compartments, like cercaria (lane 2, Figure 4.2a), which showed cross-reactivity with protein at MW around 40kDa. Trying to link these molecular weights with proteomics studies done on schistosome proteins, several proteins were allocated between 40 and 55kDa. In those proteins, enzymes such as enolase (46.9kDa), putative cytosol aminopeptidase (56.4kDa), and one similar to citrate synthase (47.9kDa) were identified (Knudsen et al. 2005; Curwen et al. 2004). Similarly, many proteins were identified around MW 30kDa, such as triosephosphate isomerase (28.1kDa), pancreatic elastase precursor (elastase 1a) (28.5kDa), elastase (elastase 1b) (29.5kDa), and one similar to carbonyl reductase (30.6kDa) (Knudsen et al. 2005; Curwen et al. 2004). Cercarial elastase, which is a serine protease, has a molecular weight near the band that showed cross-reactivity with the antivenom in the cercaria sample.

Moving to the adult stage (lane 3 Figure 4.2a), EchiTAb G antivenom showed cross-reactivity to more proteins compared with the cercaria and schistosomula stages. Six protein bands between MWs 28kDa and 50kDa, and one around 20kDa, displayed immune cross-reactivity. According to Losada et al. (2011), many proteins migrate to these molecular weights. Of those, enolase (47.4kDa), actin-2 (41.9kDa), triosephosphate isomerase, fructose-bisphosphate aldolase (39.96kDa), and thioredoxin peroxidase (21.9kDa), as well as Sm21.7 antigen (21.7kDa), were around MW 20 kDa. According to the literature (Losada et al. 2011; Curwen et al. 2004; Knudsen et al. 2005; Delcroix et al. 2007), no serine protease or metalloprotease has been identified in the adult schistosome stage within these molecular weights. This caught our interest as this may imphasize on the presence of antibodies against non-enzymatic components and non-toxic proteins in antivenom.

Two soluble egg antigen (SEA) (lane 4, Figure 4.2a) proteins reacted with EchiTAb G at MWs 30kDa and 50kDa. When locating these bands according to the SEA proteome, we found the following enzymes identified adjacent to cross-reacted bands we observed: enolase (46.9kDa), glutathione S-transferase (28kDa), and purine-nucleoside phosphorylase (31.1kDa) (Cass et al. 2007). Finally, it was difficult to detect proteins immunoreactive to EchiTAb G antivenom in the *F.*

hepatica sample (lane 5, Figure 4.2a), as smearing in the background of the sample affected the result.

Polyspecific EchiTAb-PLUS-ICP

Parasite protein cross-reactivity to the polyspecific EchiTAb-Plus-ICP antivenom was substantially different from that to the monospecific EchiTAb G antivenom.. First of all, there was a complete loss of bands at the high MWs in the cercaria sample (lane 1, Figure 4.2b) and the appearance of new low MW proteins at approximately 20kDa and 22kDa - their size perhaps indicating they might be the Sm21.7 antigen 21.7kDa, thioredoxin peroxidase 21.9kDa or glutathione S-transferase 23.8kDa (Losada et al. 2011)). In contrast, schistosomula proteins (lane 2, Figure 4.2b) displayed more reactivity toward the antivenom, and many bands at both low and high molecular weights were detected, although there was some smearing. On the other hand, the adult stage (lane 3, Figure 4.2b) cross-reacted proteins appeared in the monospecific antivenom sustained in the polyspecific EchiTAb-Plus-ICP antivenom, in addition to the appearance of a new low MW band (~18kDa), while SEA (lane 4, Figure 4.2b) lost the bands that cross-reacted, and there was smearing in the background. The polyspecific antivenom, as stated in the Methods section, was prepared from a combination of venoms from *B. arietans* (puff adder) and *N. nigricollis* (spitting cobra), in addition to *E. ocellatus*, which added antibodies to other toxins more than that present in the monospecific antivenom, such as low molecular weight neurotoxins (from *N. nigricollis* venom) and therefore, might have a wider specificity. Even in the *F. hepatica* sample (lane 5, Figure 4.2b), when compared with the monospecific antivenom cross-reactivity, we noticed an intensification of the immunoreactivity of some proteins, such as that at MW 32kDa.

Polyspecific SAIMR

This antivenom was used in this study because it was developed against a wider number of snake species (vipers and elapids) (Table 4.1) than EchiTAB-PLUS-ICP. The immunoblotting analysis revealed a different profile than found in the above results. New proteins lower than MW 25kDa appeared in cercaria, adult

Schistosoma worm, and SEA samples (lanes 1, 3, and 4, Figure 4.2c), in addition to a complete loss of schistosomula stage reactivity toward the antivenom. *F. hepatica* adult worm (lane 5, Figure 4.2c) had a single protein react, at a molecular weight of around 26kDa. This might correspond to the 25.7kDa glutathione S-transferase identified by proteomic studies on the *F. hepatica* adult worm.

Australian polyspecific CSL antivenom

Finally, we used CSL polyspecific antivenom as a control for the African antivenoms and to further investigate whether the immunoreactivity of parasite proteins to antivenoms was also evident in antivenoms developed against non-African snakes. The immunoblotting analysis revealed the existence of cross-reactivity between parasite proteins and the polyspecific antivenom, with a broad range of molecular weights, from around 20kDa to around 50kDa (Figure 4.2c).

All immunoreactivities tested in the above section were using reduced proteins. Thus the proteins have lost their secondary, tertiary, and quaternary levels of biomolecular structure, leaving only the primary structure to be analyzed. To investigate whether this immunoreactivity would be sustained when antivenoms reacted to the proteins in their native forms without any denaturing, we prepared non-reduced and native proteins from each sample and blotted against the antivenoms used in this study. For all of the above-mentioned samples, when the blotted proteins were changed from reduced to non-reduced and native, the cross-reactivity faded or disappeared, as shown in the two examples in Figures 4.3 and 4.4.

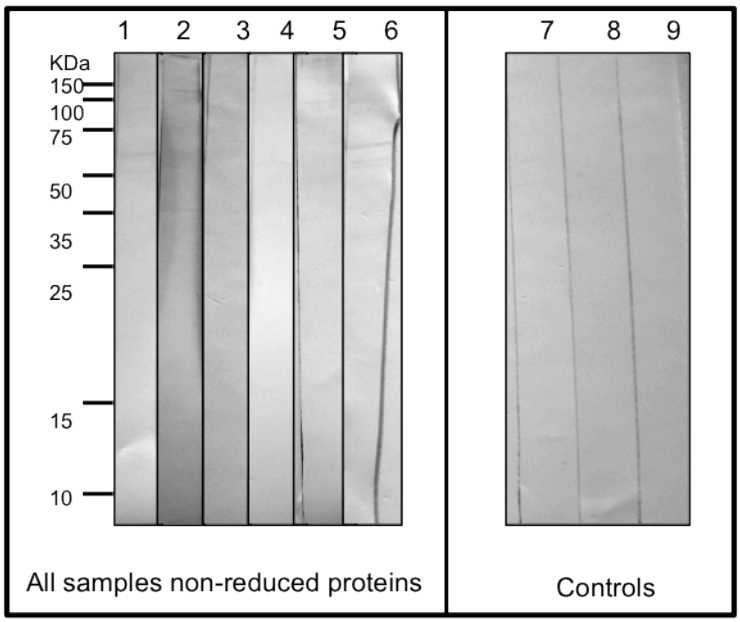


Figure 0.3 Non-reduced immunoblotting of *S. mansoni.* 1) cercaria, 2) schistosomula, 3) adult, 4) SEA, and 5) *F. hepatica* adult; against EchiTABG antivenoms and controls; 7) normal sheep, 8) anti-sheep IgG; 9) substrate only (cercaria). The molecular weight markers (kDa) are shown on the left side of the image.

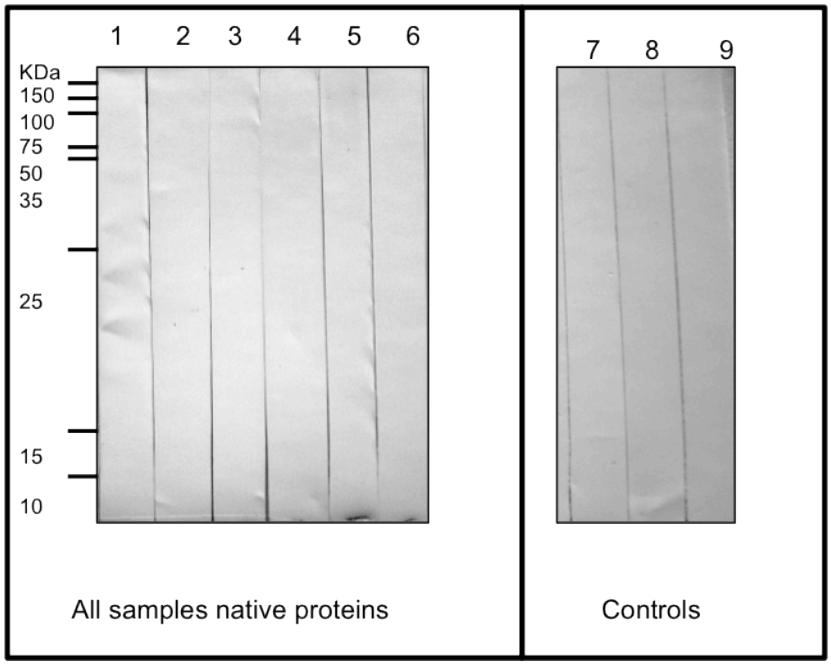


Figure 0.4 Native protein immunoblotting against EchiTAB G antivenom. 1) *S. mansoni* cercaria, 2) *S. mansoni* schistosomula, 3) *S. mansoni* adult, 4) *S. mansoni* SEA, 5) *F. hepatica* adult; 6) *An. gambiae* salivary glands and controls; 7) normal sheep, 8) anti-sheep IgG; 9) Substrate only (cercaria). The molecular weight markers (kDa) are shown on the left side of the image.

1.13.3 LC-MS/MS analysis

We conducted mass spectrometric identification to link the immune cross-reactivity we observed in the previous experiment with the transcriptomic data available for the parasites and the results we demonstrated in Chapter 3. Protein bands with significant immunoreactivity to snake antivenom were excised from the Coomassie-stained gels (Figure 4.2). The molecular weights of reactive proteins in the blots were measured and the exact protein band was excised (Table 4.2), in-gel trypsin digested then identified using bioinformatics. The functional identities of the parasite proteins that expressed cross-reactivity to the antivenom were determined. The identified proteins and the number of peptides found in each sample are listed in Table 4.3. The molecular weight and LC-MS/MS derived protein sequences of the identified proteins are also included in the table.

Among the recognised proteins, several known antigens for *S. mansoni* and *F. hepatica* were found. For example, major egg antigen p40 and fructose-bisphosphate aldolase are from the identified bands in *S. mansoni*, and glutathione S-transferase and Hsp 70 are in *F. hepatica.*

In addition, in this analysis, we identified several structural proteins that are usually recognised as part of the cell cytoskeleton, such as actin and paramyosin. Notably, all the proteins that were identified were intracellular; no extracellular structural proteins were identified.

	Cercaria	Schistosomula	Adult worm (*S. mansoni*)	SEA	*Fasciola hepatica*
EchiTAb G®	30, 50kDa	40kDa	20,28,30,40,50kDa	30, 50kDa	-
EchiTAb-Plus-ICP	20,22 kDa	-	18kDa	-	32kDa
SAIMR Polyvalent	25kDa	-	50 kDa	25kDa	26kDa
CSL Polyvalent snake antivenom	35,75, 100 kDa	35, 50, 75kDa	75kDa	35, 50, 75 kDa	50,75kDa

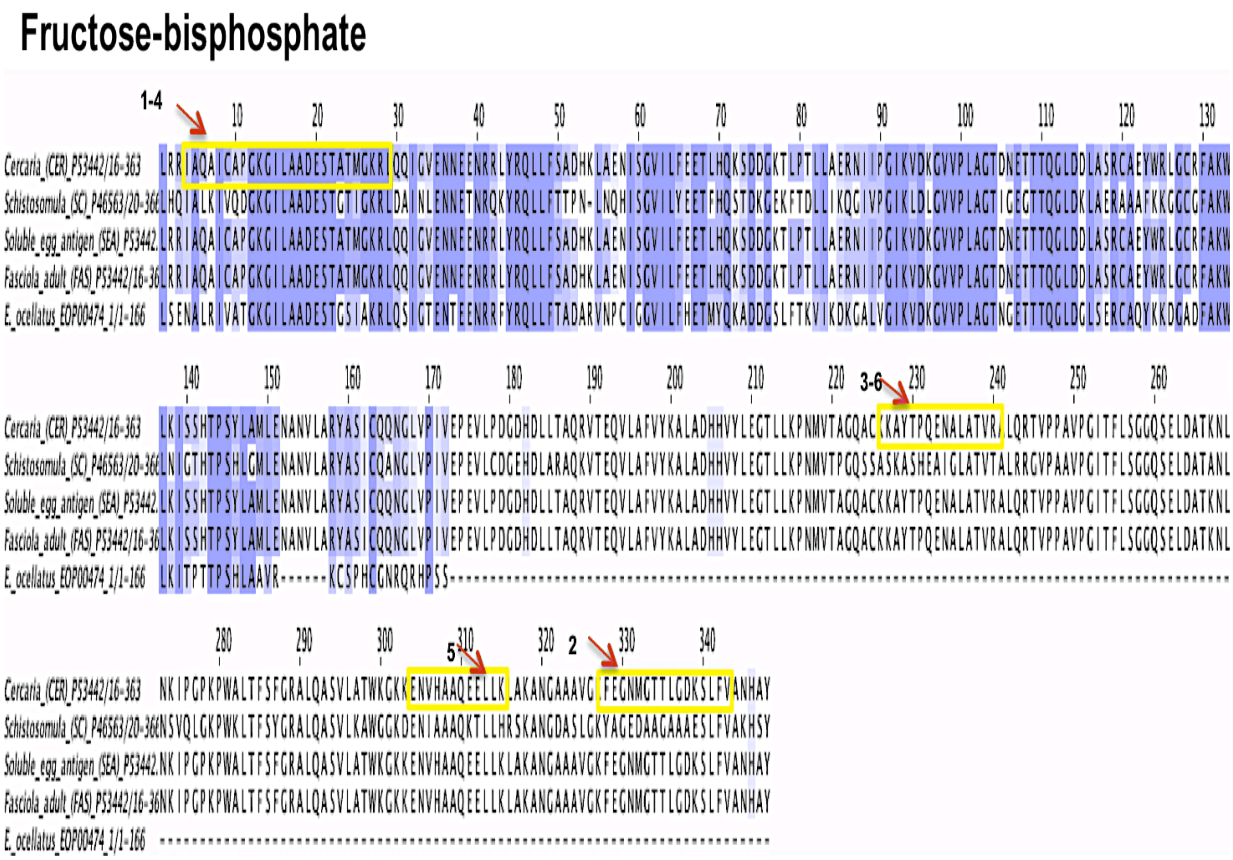
Table 0.2 Summary of positive cross-reacted protein bands from the different samples with a variety of snake antivenoms.

1.13.4 Alignment with snake venom sequences

The amino acid sequences of the identified proteins were examined for homology with those of snake venom proteins. The greatest homology was found among actin from CTF, SEA, and snake proteins. In addition, homologies were demonstrated with other proteins with varying degrees of similarity; for example, schistosoma adult worm calreticulin precursor and ECO00071_1 from snake venom, and enolase from schistosomula and alpha-enolase from snake venom. The snake venom protein EOP00474_1 sequence could also be aligned with fructose-bisphosphate aldolase from cercaria, schistosomula, SEA, and *F. hepatica* adult worm proteins. Table 4.3 and Figure 4.6 illustrate some of the identified proteins, their band size, the antivenom they reacted to, and highlights of the identified peptides by mass spectrometry.

Protein	Band MW	Actual MW	Anti-venom	Organism
Fructose-bisphosphate aldolase	35 kDa	39.6 kDa	CSL	Cercaria
Enolase	50 kDa	47 kDa	SAIMR, CSL	Schistosomula
GAPDH	35 kDa	36.4 kDa	CSL	Schistosomula
Calreticulin precursor	50 kDa	45.4 kDa	EchiTAb G, CSL	Schistosoma adult
Heat shock 70	50 kDa	69.8 kDa	EchiTAb-Plus-ICP, EchiTAb G, CSL	Fasciola SEA
14-3-3	28 kDa	28.4 kDa	EchiTAb G	Schistosoma adult

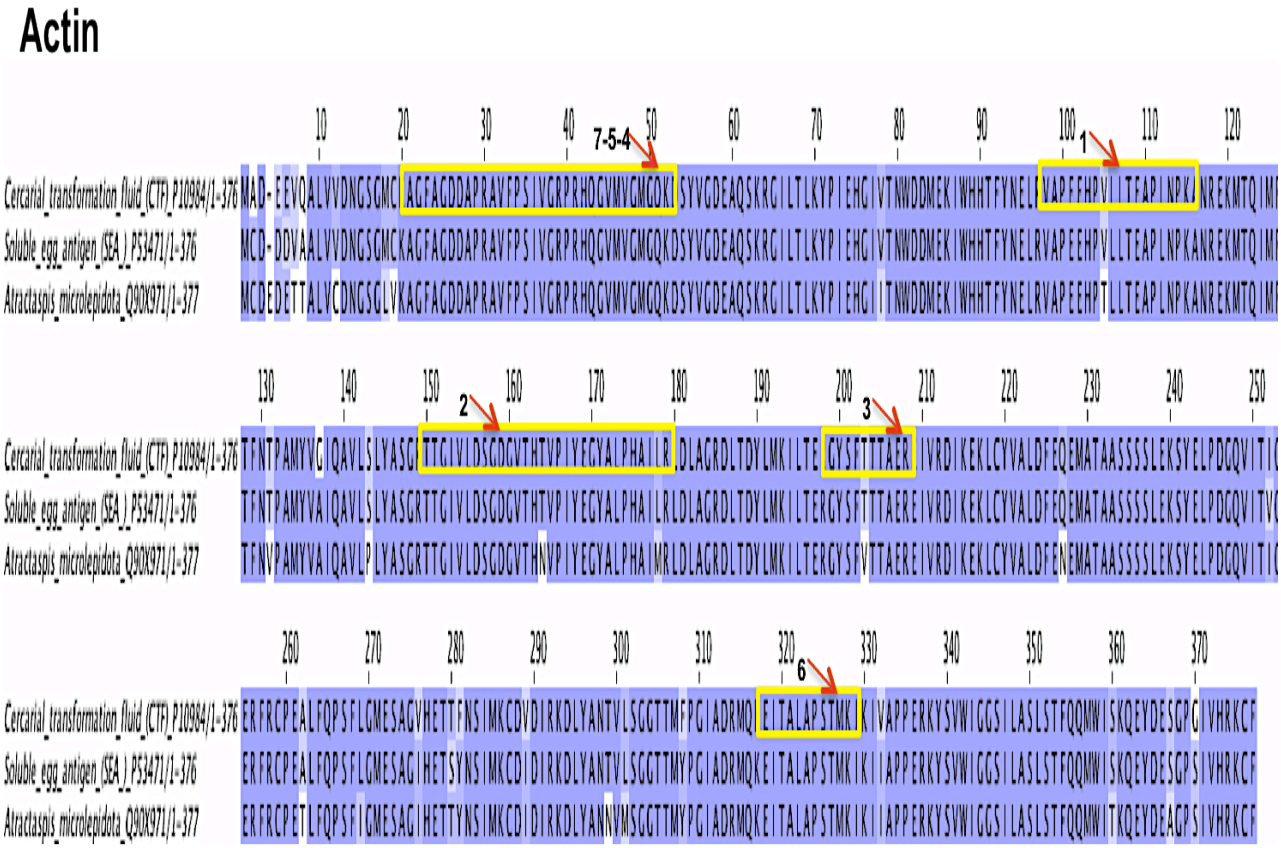
Table 0.3 Identified proteins with high coverage rates in LC-MS/MS and high level of identity with venom proteins. Actual and excised band molecular weights linked to antivenom that showed reactivity to them.



Description	Max score	Total score	Query cover	E value	Identity	Accession
Cercaria (CER) P53442	190	190	43%	2e-63	60%	152199
Schistosomula (SC) P46563	171	188	46%	6e-56	58%	152199
Fasciola adult (FAS) P53442	190	190	43%	2e-63	60%	152199

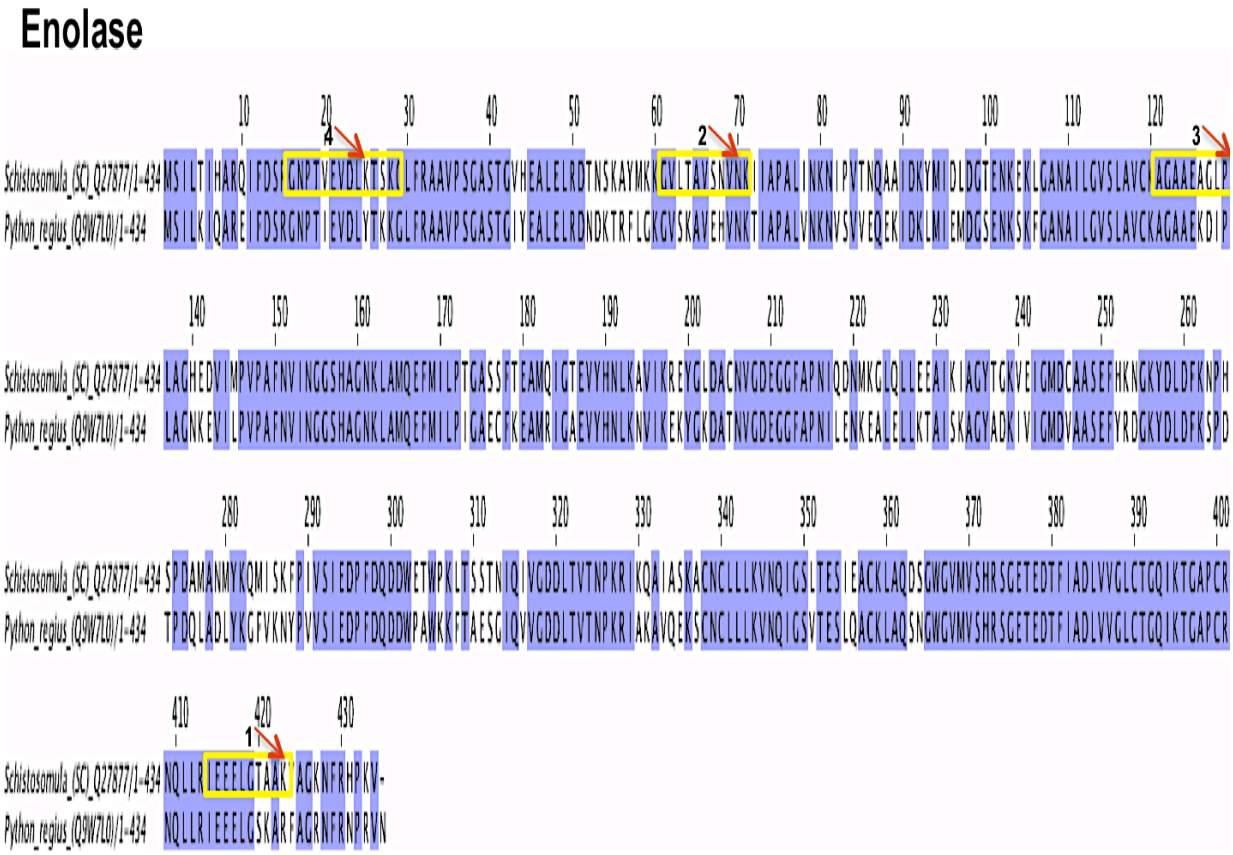
a) Sequence alignment of parasite fructose-bisphosphate aldolase identified by LC-MS/MS (CER = cercaria; SC = schistosomula; SEA = soluble egg antigen; FAS = Fasciola adult) with *E. ocellatus* matched protein. Comparative Clustal X sequence alignment of cercaria FBA (P53442), schistosomula FBA-2 (P46563), SEA FAB (P53442), and fasciola FAB (P53442) with (EOP00474_1).

Key: Yellow boxes indicate peptides identified by LC-MS/MS. Numbers and red arrows link the identified peptides with peptide column number in Table in Appendix II. The table shows identity % and E value of aligned sequences.



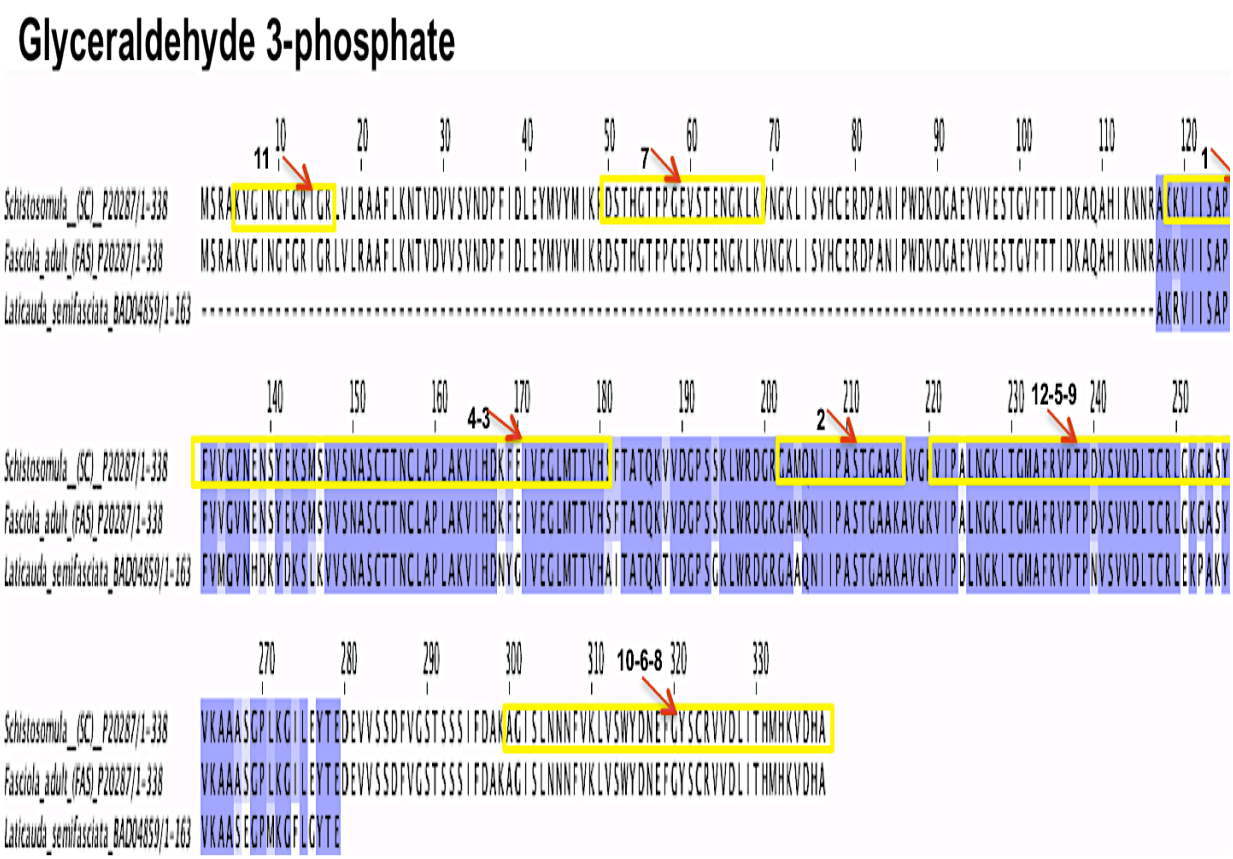
Description	Max score	Total score	Query cover	E value	Identity	Accession
Cercarial transformation fluid (CTF) P10984	747	747	100%	0.0	94%	27893
Soluble egg antigen (SEA) P53471	739	739	100%	0.0	93%	27893

b) Sequence alignment of parasite actin identified by LC-MS/MS (CTF = cercarial transformation fluid; SEA = soluble egg antigen) with *Atractaspis microlepidota* matched protein. (Note: This venom protein was used because no homologous sequence was found in *E. ocellatus* database.) Comparative Clustal X sequence alignment of CTF actin-2 (P53471) and SEA actin-2 (P10984) with Q90X971. Key: Yellow boxes indicate peptides identified by LC-MS/MS. Numbers and red arrows link the identified peptides with peptide column number in Table in Appendix II. The table shows identity % and E value of aligned sequences.



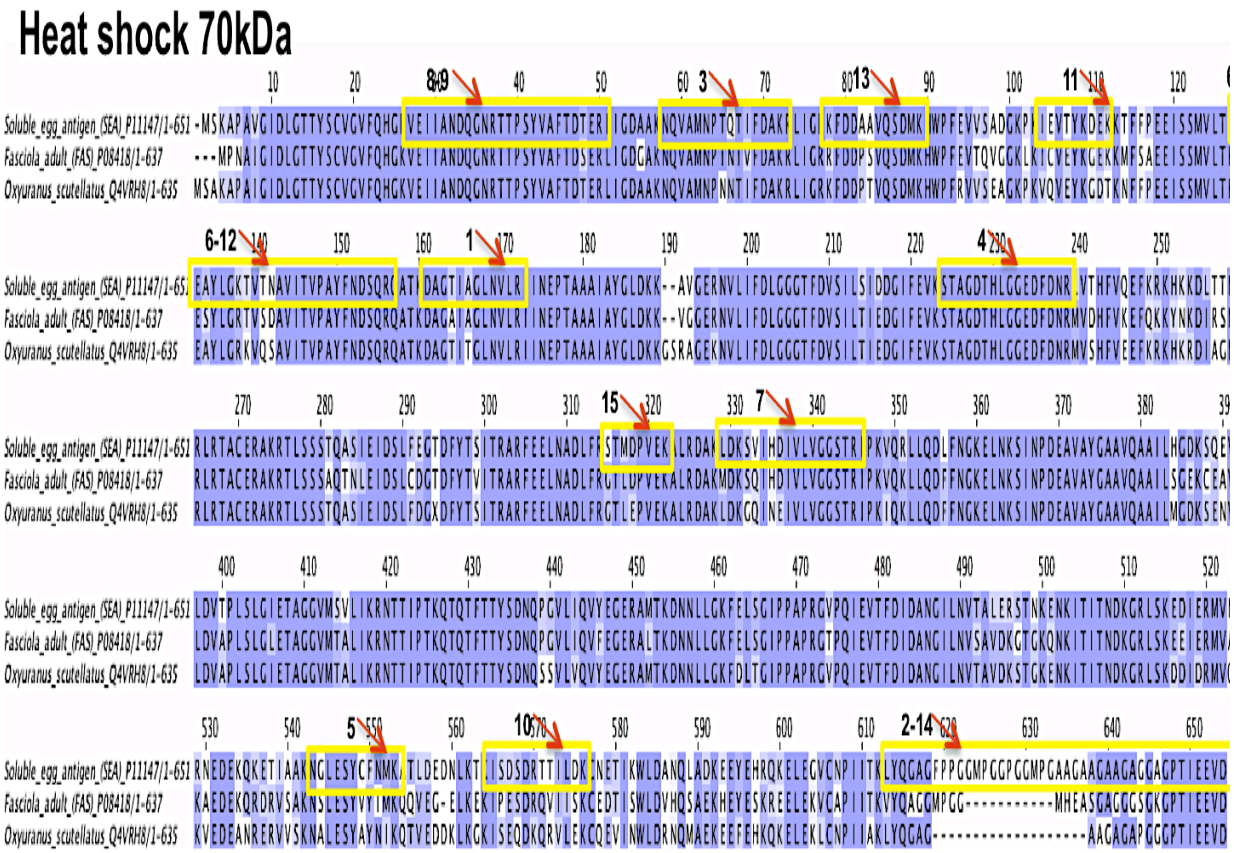
Description	Max score	Total score	Query cover	E value	Identity	Accession
Schistosomula (SC) Q27877	672	672	100%	0.0	72%	224277

c) Sequence alignment of parasite enolase identified by LC-MS/MS (SC = schistosomula) with matched protein from *Python regius* snake venom. (Note: This venom protein was used because no homologous sequence was found in *E. ocellatus* database.) Comparative Clustal X sequence alignment of schistosomula enolase (Q27877) with *Python regius* alpha-enolase (Q9W7L0). Key: Yellow boxes indicate peptides identified by LC-MS/MS. Numbers and red arrows link the identified peptides with peptide column number in Table in Appendix II. The table shows identity % and E value of aligned sequences.



Description	Max score	Total score	Query cover	E value	Identity	Accession
Schistosomula (SC) and Fasciola adult (FAS) P20287	274	274	48%	2e-96	82%	42219

d) Sequence alignment of parasite glyceraldehyde 3-phosphate dehydrogenase identified by LC-MS/MS (SC = schistosomula and FAS = Fasciola adult) with *Laticauda semifasciata* matched protein. (Note: This venom protein was used because no homologous sequence was found in *E. ocellatus* database.) Comparative Clustal X sequence alignment of schistosomula GAPDH (P20287) and fasciola GAPDH (P20287) with *Laticauda semifasciata* GAPDH (BAD04859). Key: Yellow boxes indicate peptides identified by LC-MS/MS. Numbers and red arrows link the identified peptides with peptide column number in Table in Appendix II. The table shows identity % and E value of aligned sequences.



Description	Max score	Total score	Query cover	E value	Identity	Accession
Soluble egg antigen (SEA) P11147	1065	1065	93%	0.0	83%	21627
Fasciola adult (FAS) P08418	1025	1025	95%	0.0	81%	171041

e) Sequence alignment of parasite heat shock 70kDa identified by LC-MS/MS (SEA = soluble egg antigen; FAS; Fasciola adult) with O*xyuranus scutellatus* matched protein. (Note: This venom protein was used because no homologous sequence was found in *E. ocellatus* database.) Comparative Clustal X sequence alignment of SEA HSP70 (P11147) and fasciola HSP70 (P08418) with O*xyuranus scutellatus* HSP70 (Q4VRH8). Key: Yellow boxes indicate peptides identified by LC-MS/MS. Numbers and red arrows link the identified peptides with peptide column number in Table in Appendix II. The table shows identity % and E value of aligned MS/MS.

Figure 0.5 a-e Alignment of some LC-MS/MS identified schistosome and fasciola proteins with significant immunoreactivity to snake antivenom and snake venom protein.

1.13.5 Immunoblotting with toxin-specific IgG

Immunoblotting of parasite proteins with antivenoms revealed variations in parasite immunoreactivity towards the antivenom. However, mass spectrometric identification was surprising, as none of the identified proteins were of proteases we are interested in i.e. serine proteases, metalloprotease, cysteine proteases or toxin proteins known to be neutralize by antivenom and have parallels in parasites such as PLA_2 and c-type lectin. Antivenom is produced by immunising adult horses or sheep with snake venom/s, and their immunoglobulin is purified for antivenom production. Taking into account that these animals have mature immune systems and are exposed to daily antigenic stimuli while grazing in open fields, it is not surprising that only 10% of the immunoglobulin in antivenom binds to snake venom proteins (Harrison et al. 2011). Thus, to eliminate the possibility that the parasite-reactive antivenom IgGs were stimulated by environmental antigens we tested antibodies from mice immunised with immunogens representing the following venom toxin proteins: snake venom metalloprotease SVMP (two types of IgGs—one raised against SVMP subclass P-I and the other against subclass P-II); serine protease SP; phospholipase A_2 PLA_2; and the non-enzymatic toxin proteins c-type lectin (CTL I, II—the numbers represent different domains of the protein) and cysteine-rich protein CRISP (IgGs designed and prepared at Alistair Reid Venom Research Unit, LSTM, as part of Camila Rinjifo's PhD project) (Figure 4.7).

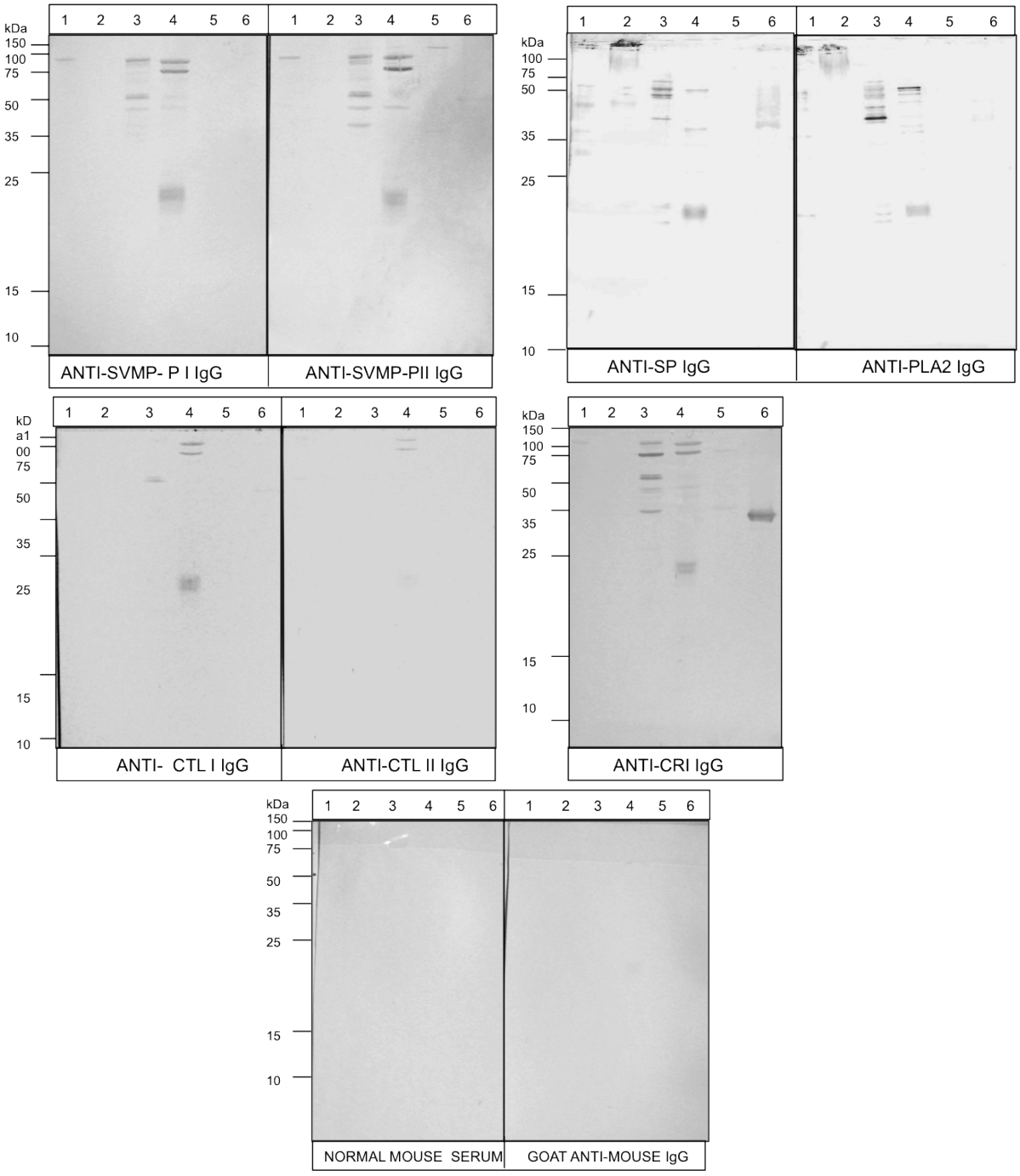


Figure 0.6 Reduced immunoblotting (15% SDS-PAGE gel) against various toxin-specific IgGs and controls: snake venom metalloprotease (SVMP PI, PII); serine protease SP; phospholipase A_2 PLA_2; c-type lectin (CTL I, II); cysteine-rich protein CRI. Samples are: 1) *S. mansoni* cercaria, 2) *S. mansoni* cercarial transformation fluid, 3) *S. mansoni* schistosomula, 4) *S. mansoni* adult, 5) *S. mansoni soluble egg antigen*, 6) *F. hepatica* adult. The molecular weight markers (kDa) are shown on the left side of the image.

The results demonstrated in this section prove that antibodies produced against toxin-specific proteases can specifically cross-react with parasite proteins.

1.14 Discussion

Shared molecules among organisms is an anticipated finding, as many molecules, such as enzymes, hormones, and receptors, have been found to be conserved during evolution (Losada et al. 2005). This has significance in the identification of molecules with potential for the development of drugs or vaccines effective against different species or genera of organisms. Comparing the cross-reactivity of parasite proteins to antivenoms known to inhibit the toxicity of proteinases in snakebite victims produced interesting findings. Different antivenoms developed to neutralize venom toxins, whether to single snake venom (monospecific) or to a group of snake venoms from different species and genera (polyspecific), showed an ability to react with parasite proteins. It was interesting to us to determine whether the presence of similar proteins in parasites and snake venom that exhibit similar functions *in silico* would reflect this *in vitro*. In migrating stages of Schistosome and Fasciola, it is known that proteases of serine protease and metalloprotease play a pivotal role in infection initiation and persistence. Therefore, we started the experiments by testing the ability of monospecific antivenom, EchiTAb G, developed against snake venom (*E. ocellatus*) known to cause major pathology using serine proteinases and metalloproteases. Comparing cross-reactivity of this antivenom toward venom with what we observed with parasites, we were expecting reactivity to proteins with molecular weights around 30kDa (i.e. serine protease) or 55kDa (i.e. metalloprotease), or at a low molecular weight of around 14kDa (i.e. PLA_2). Our observation was close to what we predicted, as we detected proteins with molecular weights around 30–50kDa in different stages of the Schistosoma parasite. We broadened the antivenom specificity, using polyspecific antivenom EchiTAb-PLUS-ICP and SAIMR polyvalent, aiming to include more IgGs to different toxin proteins in our investigation with parasites. Using these antivenoms, we observed an increase in the number of reacted proteins in some parasite stages, the disappearance of others, and the occurrence of proteins at lower molecular weights than observed with the monospecific antivenom. This variation occurred when we were investigating immunoreactivity toward antivenom of non-African snake venom. Some immunoreactive proteins appeared in more than one immunoblot, and what was interesting is that they were within the molecular weights of the proteins we are

looking for. As these antivenoms were capable of cross-reacting with parasite proteins *in vitro*, we wondered whether it would apply *in vivo*. However, before answering this question, it was necessary to identify these immunoreacted proteins and detect the specificity of the reaction. Mass-spectrometry was used to determine which proteins show cross-reactivity.

The major proteinases released from invading and transforming schistosome cercariae are known to be serine proteinases (Ingram et al. 2012; Knudsen et al. 2005). Several of the parasite serine proteinases have been characterised, and a membrane-anchored enzyme has been reported (Coombs and Mottram, 1997). We were hoping that some of the antivenoms might cross-react with this crucial enzyme, as it showed some similarity when aligned with snake venom serine proteinase, as shown in Chapter 3 (Figure 3.2). We were also hoping to see antivenom cross-reactions with the metalloproteinases involved in a variety of critical processes, including haemoglobin digestion, surface membrane re-modelling, host cell interactions, and degradation of the extracellular matrix.

However, analysing the bands that showed reactivity to antivenom was surprising; none of the identified proteins were of the protease or protease inhibitor nature we expected based on our bioinformatics results. The LC-MS/MS-identified proteins included major egg antigen p40 and fructose-bisphosphate aldolase from *S. mansoni*. Interestingly, one of the identified bands corresponded to protein 14-3-3 in the schistosome adult. In parasites, this protein could be involved in different processes, such as interaction between sexes and sexual maturation, interaction with the host, excretion, and movement (Schechtman et al. 2001; Liu et al. 2009; El Ridi and Tallima 2009). When regarding the same protein in snake venom, the literature describes a relation between 14-3-3 protein and snake venom PLA_2s. The protein was found to act as an acceptor for the neurotoxic PLA_2 (Šribar et al. 2003).

In the MS analysis, we identified several structural proteins that are usually recognised as part of the cell cytoskeleton, such as actin and paramyosin. Notably, all the proteins that were identified were intracellular; no extracellular secreted proteins were identified. These finding emphasises that a similarity in transcription does not necessarily correlate with protein expression. It should also be kept in mind that the samples we used were from whole parasite stage homogenates and not secreted proteins only. In addition, it is important to remember that

we investigated cross-reactivity between very distinct organisms and using antivenom developed against whole venom that contained non-toxic components in addition to toxin proteins.

To exclude any non-specific cross-reactivity that might result from the fact that the antivenom was developed against whole venom protein composed of a mixture of other proteins in addition to toxins, we tested the ability of toxin-specific IgGs to cross-react with parasite samples. We selected IgGs developed against several snake venom toxin proteases: anti-SVMP PI, produced to bind to the 25–30kDa metalloprotease PI in vipers; anti-SVMP PII 30–45kDa; anti-SP that binds to the 26–47kDa thrombin-like serine proteases in vipers; anti-PLA_2 that immunoreacts with the low MW 13–18kDa snake venom PLA_2; and two IgGs developed against non-enzymatic component c-type lectin 26–28kDa and cysteine-rich secretory proteins 21–25kDa (venom toxin MWs according to Mackessy, 2010). Marked cross-reactivity was detected against several toxin-specific IgGs: e.g. anti-SVMP PI, PII; anti-SP; anti-PLA_2; anti-CTL; and anti-CRI I. Interestingly, the different schistosome life-stages exhibited variable reactivity profiles to toxin-specific IgGs. For example, as Figure 4.7 illustrates, cercaria cross-reacted with anti-serine protease IgG (SP) in two different bands of MW 28 and 45 kDa, which, while necessarily speculative, may represent cercarial elastase and another serine protease from this parasite's stage. In addition to that cross-reaction, the cercaria sample showed some reaction to SVMP PI, an IgG developed against a specific domain of snake venom metalloprotease, which again might represent a metalloprotease used at this stage to penetrate the skin or avoid the immune system. It is possible that this variation in immunoreactivity is related to the expression of these proteins in each stage and to the functions they perform during the journey from the site of infection to the site of pathogenesis. It would have been beneficial if we could have obtained LC-MS/MS identification to confirm the identities of the proteins. However, as mentioned previously, the parasite sample size and the time frame did not allow us to perform this experiment.

Overall, although using samples of only parasite secretory proteins may provide more substantial identifications, the results observed in this chapter indicate the possibility of significant cross-reactivity of antivenom to parasite proteases. This directs our attention towards whether a similar immunoreactivity towards snake venom proteins exists in parasite-infected

patients, and if so, to what extent the immunoreactivity can be sustained. Investigation of this question will be addressed in the next chapter.

Chapter 5 Examining Immunoreactivity to Various African Snake Venoms of Sera from Parasite-Infected Humans in Africa

1.15 Introduction

Parasite infection is a constant immunological and clinical challenge to host immune systems, as parasites consume host resources and affect immune responses. Extensive evidence for the influence of parasites on various pathways of the immune system has been deliberated. Chronic helminth and trematode infections are characterised by the distortion of immune reactions towards the T helper 2 (Th2) type response, including the upregulation of IL-4, IL-5 and IL-13 cytokine production, which induces B-lymphocytes to shift to IgE antibody production, as well as to regulatory responses (Riveau and Capron, 2005). Attenuation of the regulatory network that prevents augmented immune responses facilitates the long-term survival of parasites in the host. Presumably, modulation of the host immune system is beneficial to the parasite, as it might prevent their eradication and, at the same time, protect the host from extreme proinflammatory responses (El-Ansary, 2003). Hence, immune hyporesponsiveness is frequently apparent in chronic parasitic infections (Zandman-Goddard and Shoenfeld, 2009). During infection, the immune system is first triggered to try to eradicate the parasite; however, as the burden or time after infection increases, the parasite seems to be able to modify and downregulate these specific responses in order to survive (El-Ansary, 2003).

These modifications not only change the immune system towards the parasites; they have more generalised effects towards even non-infectious diseases. Studies have demonstrated that the presence of parasitic infections might have a protective effect in murine models of particular autoimmune diseases (Zandman-Goddard and Shoenfeld, 2009). For example, the development of type 1 diabetes in non-obese diabetic (NOD) mice is inhibited by prior exposure to *Schistosoma mansoni* eggs or to soluble extracts from either the adult worms or eggs (Zandman-Goddard and Shoenfeld, 2009). In addition, helminths have been shown to develop protection in several models of inflammatory bowel disease (IBD), where the disease and the parasites are co-localised. A concurrent *S. mansoni* infection in a semi-permissive rat model decreased the course of colitis, and *S. mansoni* was shown to exhibit a protective effect in experimental autoimmune encephalitis (EAE), a murine model for multiple sclerosis (Zandman-Goddard and Shoenfeld, 2009).

Taking into account that most African populations at risk of snakebite reside within parasite endemic areas, and adding in the above-mentioned factors and the turbulence in the immune system caused by parasitic infections towards even non-infectious diseases, raises the question of whether this situation can be applied to immunity towards snake venom.

In examining this type of immunity, we found that human immunity against snake venom is a debatable topic (Isbister et al., 2010). There is limited verification supporting the development of human antibodies to snake toxins after repeated exposure. In some studies that have measured IgG levels after a second exposure of patients to the same snake venom, the patients had an elevated IgG titre against the venom, but it failed to protect them from local injuries and myocytosis (Isbister et al., 2010; Theakston et al., 1981). Ethical concerns have prevented any studies on the ability of venom-stimulated antibodies to protect against envenoming effects; however, there have been several anecdotal reports from snake handlers to suggest that self-immunisation with venom stimulates antibodies, and that some individuals exhibit a measure of protection against envenoming, although many do not. There is no immunological reason to suggest that humans could not develop the same level of venom-specific IgG if they are subjected to the same venom-immunisation program as the horses or sheep involved in antivenom production.

From a pathological point of view, the local and systemic effects of snakebite are often characterized by proinflammatory responses. The injection of the venom components into the victim's tissues, and the direct pathological effects of the toxins provoking necrosis and haemorrhage, stimulate a prominent and complex inflammatory reaction associated with the synthesis and release of inflammatory mediators, followed by the recruitment of leucocytes (Gutiérrez et al., 2007; Farsky et al., 2005; Harrison et al., 2011). The anti-inflammatory effects of helminth infections might, through this immunological route, also impart a level of tolerance to the effects of snakebite.

There are many ways to answer the broad question asked previously about whether the influence of a parasitic infection can favour the host immunity toward snake venom. However, by going through the progression of this study and correlating the findings in the literature with our findings, the objective of this chapter became to examine whether anti-helminth parasite

IgGs reacted with snake venom proteins, and if the presence of cross-reactive antibodies to snake venom in patients with parasitic infections imparts a level of protection to snakebite that has not before been recognized. In addition, investigate the ability of therapeutic IVIG to neutralize venom toxins and compare if these IVIG purified from malaria-infected patients will have a better affinity binding to snake venom toxin proteins.

1.16 Materials and Methods

The materials (parasite samples, venom, immunoglobulins and sera) described in this chapter were collected and prepared as described in Chapter 2.

1.16.1 Human sera and immunoglobulins

For the experimental investigations in this chapter, the following were used:

- Serum collected from patients with schistosomiasis
- Sera from patients with symptomatic and asymptomatic filariasis infections;
- Malawian malarial patients' IgGs;
- IgGs purified for therapeutic reasons; GAMMAGARD (IVIG)
- IgM ; PENTAGARD.

These human products were selected according to the following criteria: first, for the parasite infected sera and IgGs, they were all purified from African individuals living in areas at high risk for snakebite. All of them were tested in other studies for antibodies against parasites and all were positive.

The Malawian IgGs were purified from malaria-infected patients, selected because the literature specified that Africans infected with parasitic diseases have a provoked

hypergammaglobulinemia, as a result of the persistent presence of antigens (Mekhaiel et al., 2011). Thus, indications suggest that this IgG may be more effective at treating autoimmune diseases, rather than IVIG purified from European donors whose immune systems are not persistently being stimulated by parasites. Therefore, we wanted to examine if these IgGs are capable of neutralizing venom toxin proteins, and then compare them with GAMMAGARD and PENTAGARD purified from non-African donors.

The non-infected normal human sera and purified IgGs were all from non-African populations, American or European, and were provided by the manufacturer and stored as specified by that manufacturer (Sigma, UK). A detailed description of the sera and immunoglobulin sources and collection are described in Chapter 2, Section 2.4.

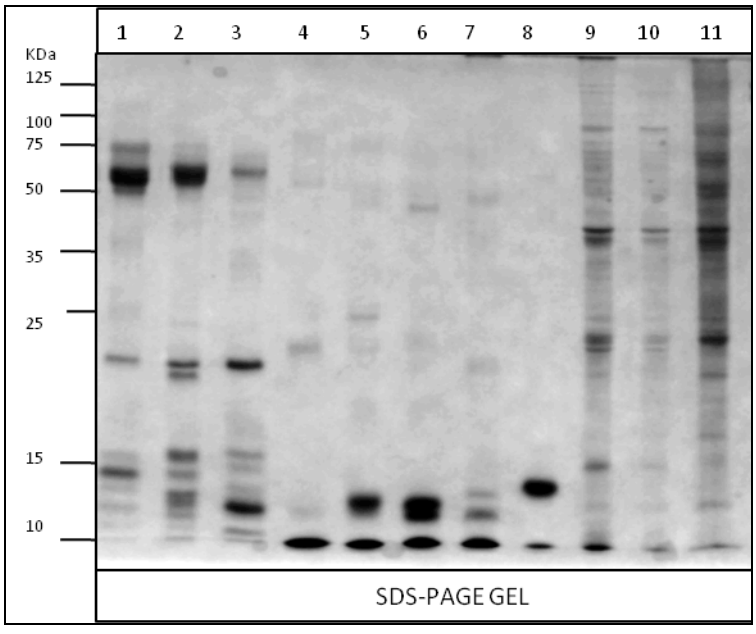
For the immunoblotting examinations, the primary antibodies (sera or immunoglobulin) were diluted in blocking buffer at a dilution of 1:100 and 1:200, and the blots were incubated overnight in the solution. The next day, the blots were incubated for 1 hour with a secondary antibody (goat anti-human IgG) (1:1000 dilution), coupled to horseradish peroxidase (HPR). Visualization of the results was performed using a 3,3'-diaminobenzidine peroxidase (DAB) substrate (Sigma, UK) after washing with TBST as described in Chapter 2, Section 2.6.

For the ELISAs, the end-point titre started from the 1:10 diluted primary antibody, followed by 1:2 serial dilutions, and the method was repeated across the wells of the plate. Related to the avidity ELISA, the plates were coated with *Echis pyramidum leakeyi* venom and incubated overnight at 4°C, with a 1:10 dilution for the immunoglobulins and 1:50 for the sera (the avidity titres were optimized according to the end-point titres and amount of sera available), as described in Section 2.8 a-b, Chapter 2.

1.17 Results

1.17.1 Reactivity of human IgG and sera to snake venom proteins

The goal of this experiment was to investigate the possibility of the presence of antibodies that are able to immunoreact with venom toxin proteins in the sera of patients infected with parasites in African provinces known to be at high risk of snakebite. The following snake venoms were selected to cover most parts of the African continent, specifically areas at high risk of both parasitic infections and snakebite: *Echis ocellatus* (West Africa), *Echis pyramidum leakeyi* (East Africa), *Bitis arietans* (spread through most parts of Africa and the southern parts of the Arabian peninsula), *Naja haje* (North and West Africa), *Naja nigricollis* (Sub-Saharan Africa), *Naja pallida* (East Africa) and *Dendroaspis angusticeps* (east coast of southern Africa). Similar to why we selected a non-African antivenom in the previous chapter, *Acanthophis antarcticus,* found in Australia, was selected to test if the reactivity was exclusive to African snake venom. As a comparison, the following parasites samples were used: *Schistosoma mansoni* cercaria, *S. mansoni* adult and *Fasciola hepatica* adult (Figure 5.1).



LANE	SAMPLE	
1	*Echis ocellatus*	Viper (snakes)
2	*Echis pyramidum leakeyi*	
3	*Bitis arietans*	
4	*Naja haje*	Elapid (snakes)
5	*Naja nigricollis*	
6	*Naja pallida*	
7	*Dendroaspis angusticeps*	
8	*Acanthophis antarcticus*	
9	*S. mansoni* cercaria	Trematode (parasites)
10	*S. mansoni adult*	
11	*F. hepatica* adult	

Figure 0.1 15% reduced SDS-PAGE profiles of eight venoms from the families' elapids and vipers, 1) *E. ocellatus*, 2) *E. p. leakeyi*, 3) *B. arietans*, 4) *N. haje,* 5) *N. nigricollis,* 6) *N. pallida,* 7) *D. angusticeps,* 8) *A. antarcticus;* and 15% reduced

SDS-PAGE profile of parasites 9) *S. mansoni* cercaria, 10) *S. mansoni* adult, and 11) *F. hepatica* adult. 5µg of protein was loaded in each well. The molecular weight marker (kDa) is shown on the left of the image.

i) Reactivity of sera collected from filariasis patients to snake venom

In the immunoblotting profiles, several protein bands, ranging in molecular weight from 10 to 125 kDa, were visible in the different antigen preparations. First, the immune-cross-reactivity to the symptomatic filariasis patients' sera was completely different when comparing the sera collected from the asymptomatic patients with that of the symptomatic individuals. By far, the viper snake venom cross-reactivity profile showed a reaction to various low and high molecular weight venom proteins, with a band of approximately 23 kDa present in all three vipers, which resembles the molecular weight of the venom toxin SVMP PI (MacKessy, 2009; Casewell et al., 2009).

The dominant immunoreactive protein in each snake was different, as illustrated in Figure 5.2. The *E. ocellatus* dominant protein had a molecular weight of around 50 kDa, which again, might be linked to the SVMP, however to a different sub-class PIII, compared with 60 and 16 kDa in *E. p. leakeyi* and *B. arietans,* which may correlate to L-amino acid oxidase (LAAO) and PLA_2, respectively (MacKessy, 2009; Mitra and Bhattacharyya, 2013). In contrast, the most dominant proteins in the elapid snake venom had low molecular weights, with 10-15 kDa protein bands being highly represented, which share a molecular weight with PLA_2 (14 kDa) and the three-finger neurotoxins (MacKessy, 2009; Fry et al., 2009; Gutiérrez & Lomonte, 2013; Bryan G Fry et al., 2003; B G Fry et al., 2003; Alape-Girón et al., 1999). However, an interesting observation is the disappearance of these highly cross-reacted, low molecular weight elapid proteins in blots probed with symptomatic filariasis patients (Figure 5.2), although these proteins were detected, even in sera collected from normal blood donors (Figure 5.4).

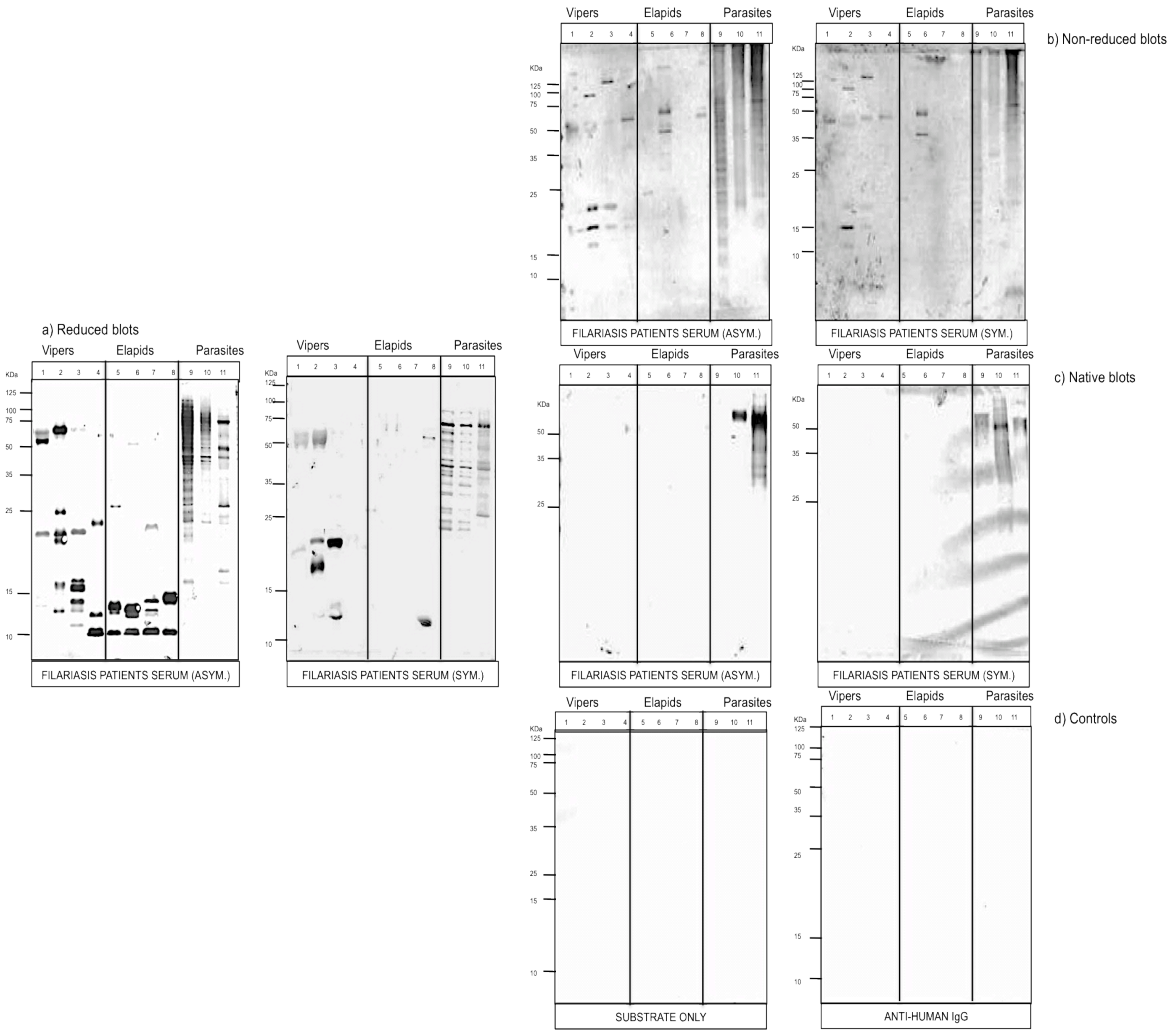


Figure 0.2 Immunoreactivity profile of filariasis patients' sera to venom proteins: 15% reduced, non-reduced, and native gel immunoblotting against filariasis patients' sera and controls at a dilution of 1:200 in the blocking buffer. Snake venoms: 1) *E. ocellatus*, 2) *E. pyramidum leakeyi*, 3) *Bitis arietans*, 4) *Naja haje*, 5) *Naja nigricollis*, 6) *Naja pallida*, 7) *Dendroaspis angusticeps,* and 8) *Acanthophis antarcticus,* and parasites: *9) S. mansoni* cercaria, 10) *S. mansoni* adult, 11) *F. hepatica* adult homogenate. The molecular weight marker (kDa) is shown on the left of the image.

ii) Reactivity of sera collected from Schistosomiasis patients to snake venom

Sera collected from patients with schistosome infections showed cross-reactivity to various high and low molecular weight proteins in vipers, and mainly low molecular weight proteins in elapids, as these proteins are the dominant proteins in these venoms – therefore the reactivity observed may not be specific (Figure 5.3). The interesting finding, again, was the loss

of reactivity with the highly antigenic, low molecular weight elapid proteins when using sera collected from the same infected patients after one year of treatment, in patients living in a village with a low prevalence of schistosomiasis (Lwanika village) (Figure 5.3). As mentioned in Chapter 2, the schistosomiasis patients' sera were collected from two villages, one with a high prevalence of infection (Bugoto), and the other with a low prevalence (Lwanika), on two separate occasions. The first was a baseline collection before treatment, and the second was one year after treatment.

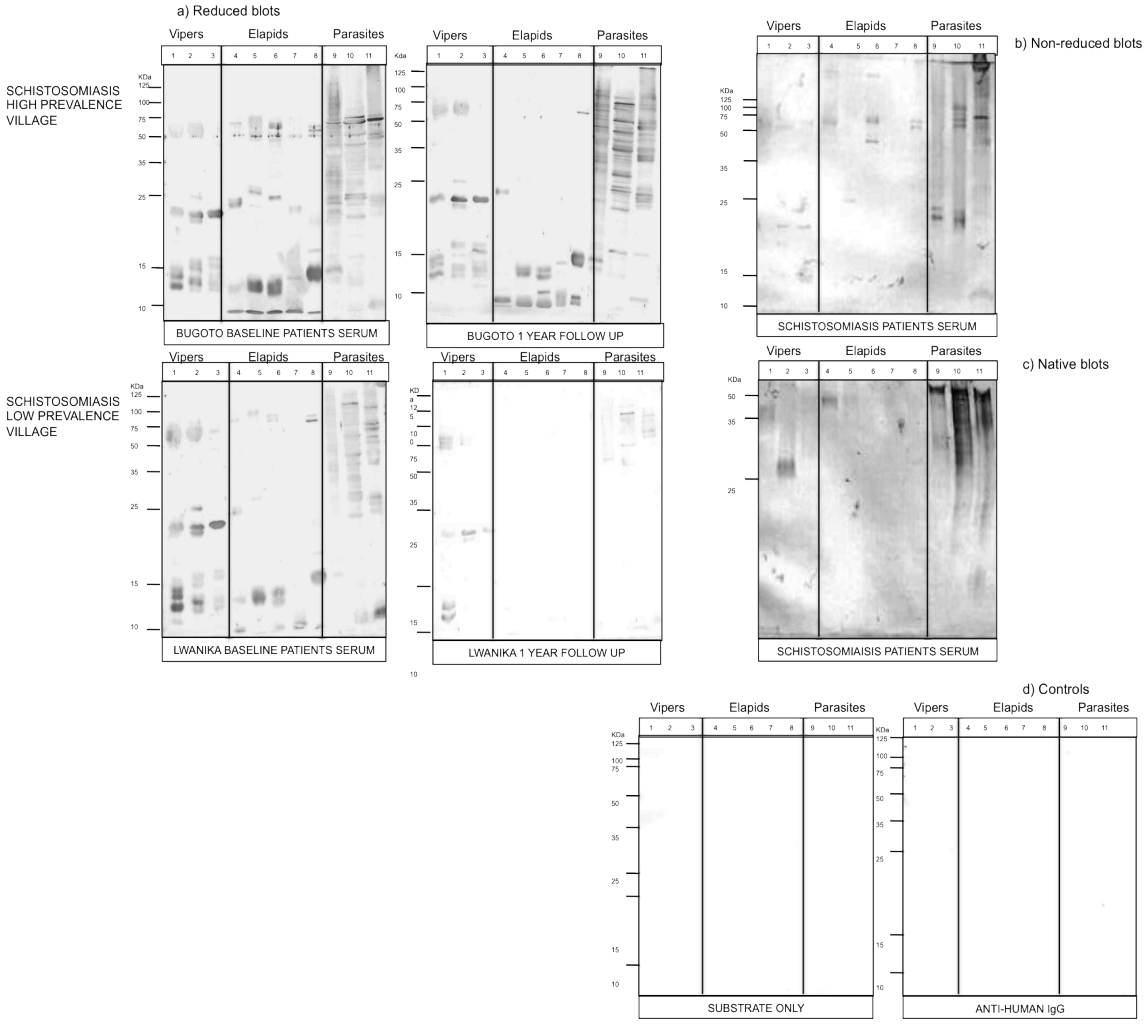


Figure 0.3 Immunoreactivity profile of schistosomiasis patients' sera to venom proteins: 15% reduced, non-reduced and native gel immunoblotting against schistosomiasis patients' sera and controls at a dilution of 1:200 in blocking buffer. Snake venoms: 1) *E. ocellatus*, 2) *E. pyramidum leakeyi*, 3) *Bitis arietans*, 4) *Naja haje*, 5) *Naja nigricollis*, 6) *Naja pallida*, 7) *Dendroaspis angusticeps,* and 8) *Acanthophis antarcticus,* and parasites: *9) S. mansoni* cercaria, 10) *S. mansoni* adult, 11) *F. hepatica* adult homogenate. The molecular weight marker (kDa) is shown on the left of the image.

iii) Reactivity of sera collected from healthy individuals to snake venom

Of note, the immunoblotting venom proteins to sera collected from healthy individuals, sterile-filtered from American males' AB plasma (Sigma), showed reactivity to similar proteins reacted with patients samples, although with a weaker reaction (Figure 5.4). We were not expecting this reactivity in healthy humans (we selected it to be a negative control), presumably the IgG-donors had not been bitten by snakes.

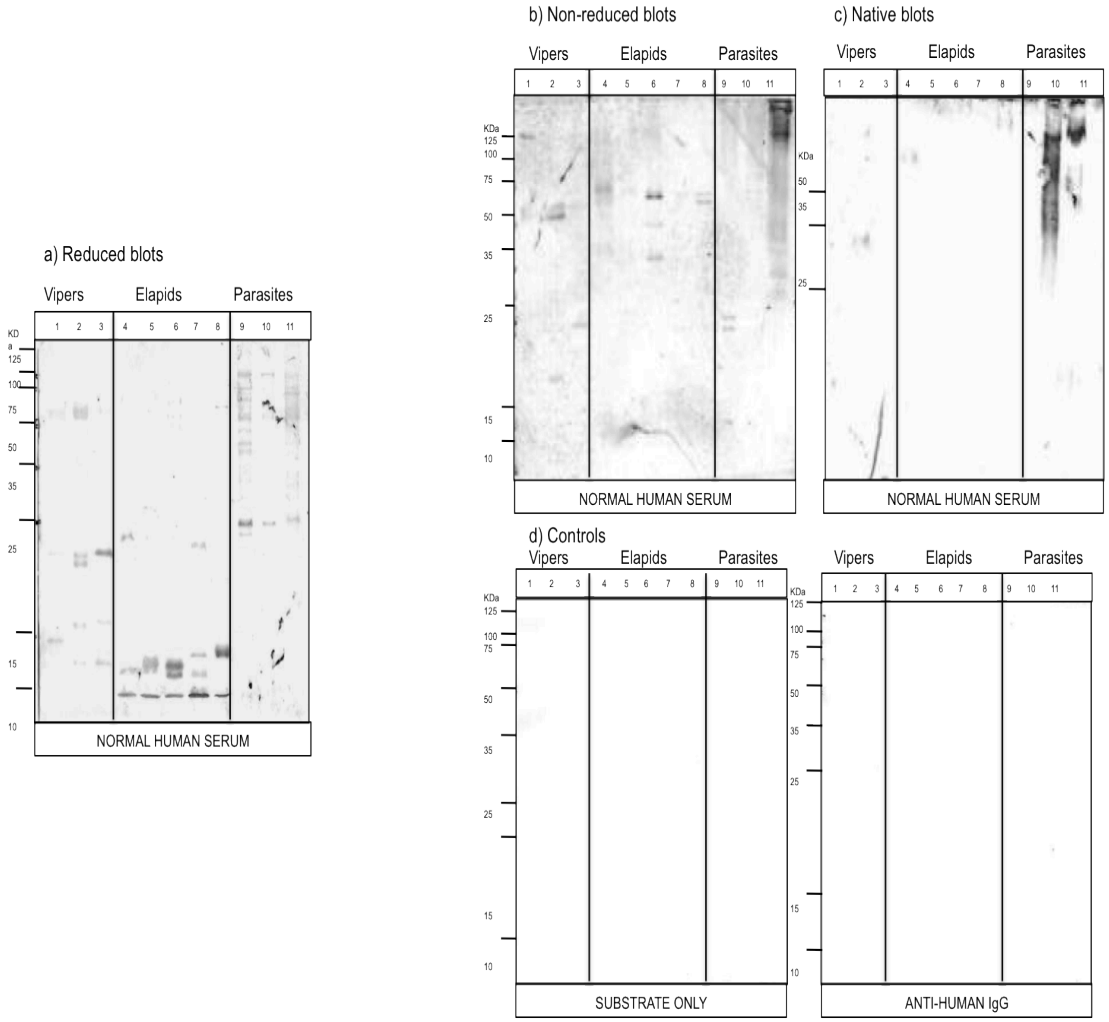


Figure 0.4 Immunoreactivity profile of normal human sera to venom proteins: 15% reduced, non-reduced and native gel immunoblotting against normal human sera, and controls at a dilution of 1:200 in blocking buffer. Snake venoms: 1) *E. ocellatus*, 2) *E. pyramidum leakeyi*, 3) *Bitis arietans*, 4) *Naja haje*, 5) *Naja nigricollis*, 6) *Naja pallida*, 7) *Dendroaspis angusticeps,* and 8) *Acanthophis antarcticus,* and parasites: *9) S. mansoni* cercaria, 10) *S. mansoni* adult, 11) *F. hepatica* adult homogenate. The molecular weight marker (kDa) is shown on the left of the image.

iv) Reactivity of intravenous immunoglobulin IVIG to snake venom

In this experiment we used purified immunoglobulins:

- IgGs purified from Malawian malaria patients
- as negative control, IgGs (IVIG) (gamma globulin)
- IgMs (penta globulin) purified from healthy donors.

Intravenous immunoglobulin (IVIG) has been used for the treatment of autoimmune inflammatory disorders (Ballow, 2011; Orange et al., 2006; Mekhaiel et al., 2011). Moreover, studies showed that IgGs collected from the African population have a mean concentration that is approximately twice that of the UK controls (Rowe et al., 1968; Mekhaiel et al., 2011). This significant increase in immunoglobulin levels appears to be the result of a notable polyclonal B and T cell activation associated with protozoal infections such as malaria and African trypanosomiasis (Mibei and Orago, 2005). Similar to IVIG, IgM showed the ability to stimulate autoantibody activity through idiotypic interactions (Hurez et al., 1997). Therefore, we wanted to test *in vitro* the capability of Malawian IgGs to cross-react with snake venom, and probably to prevent the immunopathology caused by snake venom; then compare that with the reactivity of commercially-available IVIGs prepared as IgG and IgM formulations.

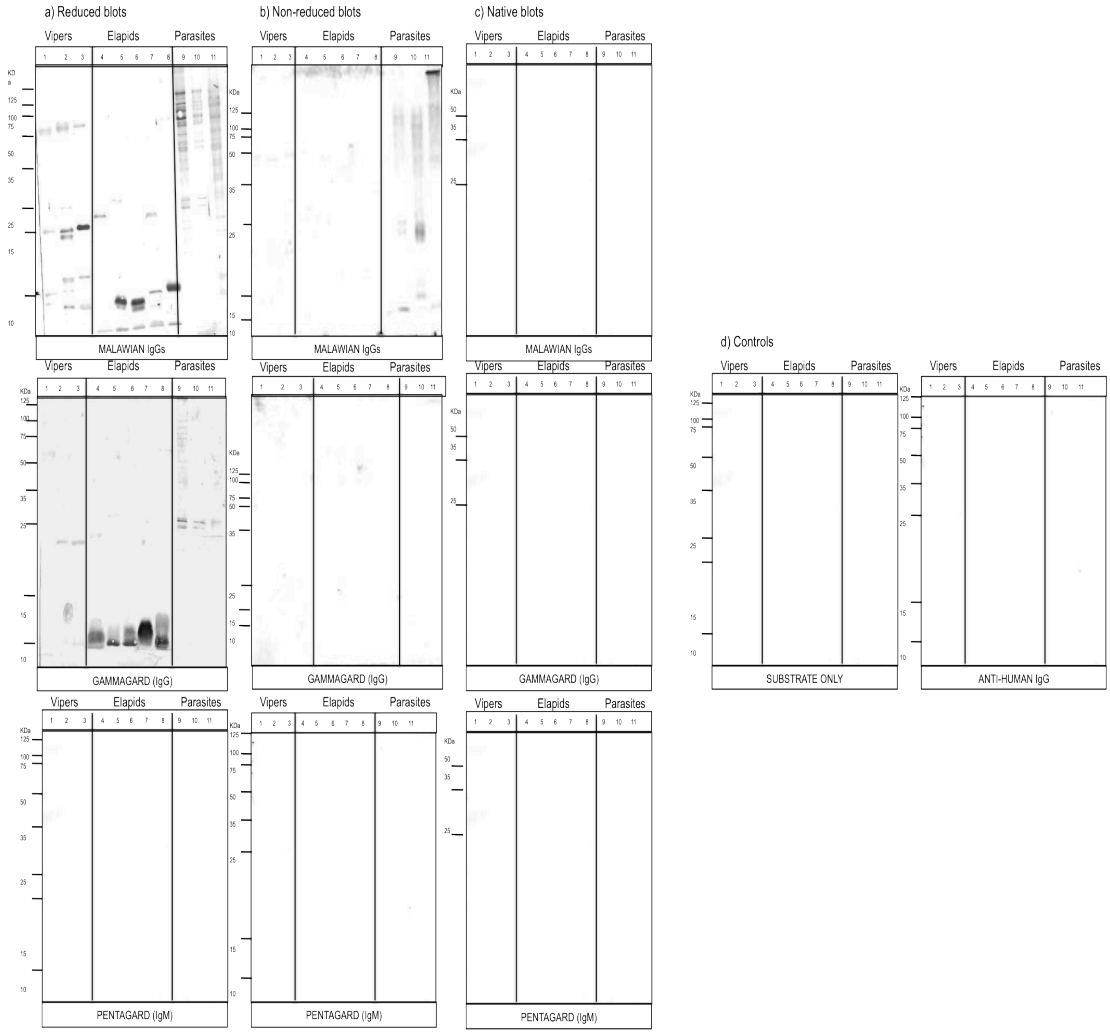


Figure 0.5 Immunoreactivity profile of purified immunoglobulins IVIG to venom proteins: 15% reduced, non-reduced and native gel immunoblotting against purified immunoglobulins IVIG and controls at a dilution of 1:200 in blocking buffer. Snake venoms: 1) *E. ocellatus*, 2) *E. pyramidum leakeyi*, 3) *Bitis arietans*, 4) *Naja haje*, 5) *Naja nigricollis*, 6) *Naja pallida*, 7) *Dendroaspis angusticeps,* and 8) *Acanthophis antarcticus,* and parasites: *9) S. mansoni* cercaria, 10) *S. mansoni* adult, 11) *F. hepatica* adult homogenate. The molecular weight marker (kDa) is shown on the left of the image.

IgGs purified from the malaria patients in Malawi exhibited intense and reactivity to the low molecular weight elapid venom antigen, compared with a more comprehensive and broad reactivity to viper venom (Figure 5.5). On the other hand, GAMMAGUARD (IVIG), filtered from normal individuals, expressed less reactivity than that showed in the Malawian IgG, with high cross-reactivity to low molecular weight elapid proteins only, and limited immunoreactivity

to proteins of approximately 21 kDa in vipers in general. In contrast, the IgMs failed to show any cross-reactivity to any of the used antigens, whether venom or parasites (Figure 5.5).

To complete the cross-reactivity profile, and to investigate whether this immunoreactivity would be continued when the sera and IVIG reacted to the proteins in their native forms: non-reduced and native proteins were processed and probed against the above-mentioned primary antibodies (Figures 5.2- 5.5). When non-reduced proteins are used, the cross-reactivity profiles change. An intense reaction occurred, with low molecular weight elapid venom antigens disappearing and a few high molecular weight proteins reappearing. However, the viper non-reduced proteins showed cross-reactivity to proteins with high and low molecular weights, similar to that which it showed in the reduced blots. On the other hand, blots probed with immunoglobulin showed very week or no reaction at all. Likewise, the native proteins failed to cross-react with any of the antibodies and sera, except for sera collected from patients living in the high schistosomiasis prevalence village (Figures 5.2-5.5).

1.17.2 Reactivity of human IgG and sera to deglycosylated snake venom proteins

In order to determine if the immunoreactivity of snake venom toward human sera and immunoglobulin is affected by post-translational glycosylation of some venom proteins, a deglycosylation experiment was done for the proteins prior to immunoblotting.

Although the PNGase F enzyme will release glycans from many glycoproteins in their native forms, the denaturation step is still required to guarantee complete hydrolysis of all subject bonds. Proteins that were run on SDS-PAGE in this experiment were in a fully denatured state.

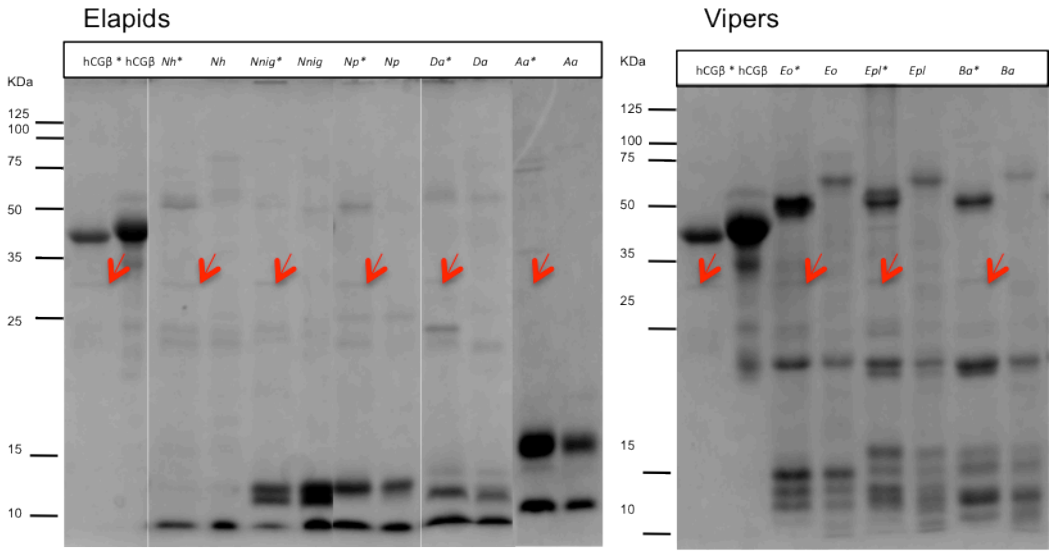


Figure 0.6 SDS-PAGE profile of deglycosylated venom proteins: 15% reduced SDS-PAGE profiles of eight venoms from the elapids: *N. haje, N. nigricollis, N. pallida, D. angusticeps* and *A. antarcticus,* and vipers: *E. ocellatus*, *E. p. leakeyi*, *B. arietans*, after *in vitro* enzymatic deglycosylation was achieved by the addition of PNGase F (10 units/mL) to 140 µg of venom proteins, with incubation for 1 hour at 37°C. An equal amount of PNGase F treated (*lane) and non-treated (next lane) lysate (10 µg) was loaded. The molecular weight marker (kDa) is shown on the left of the image. Red arrows indicate the molecular weight of PNGase F at around 36 kDa**.**

As expected, only a minor shift in electrophoretic mobility was observed in the elapid venom when compared to the non-digested venom, and there was no difference between the deglycosylated and non-deglycosylated low molecular weight proteins (Figure 5.6). However, in the viper venom, deglycosylation resulted in the appearance of new protein bands in addition to the slight shift in the other proteins, indicating how glycosylation contributes to the total protein molecular mass and antigenicity. A comparison of the digested proteins (lanes with *) and the non-digested ones (lanes without *) revealed that the deglycosylated proteins in both elapid and viper venom migrated at a slightly lower molecular weight than the glycosylated material, suggesting that deglycosylation was complete. The molecular weight of PNGase F was 36 kD (arrows in Figures 5.6 and 5.7).

Comparing the immunoreactivity profile of the deglycosylated and non-deglycosylated proteins showed a very minor difference between the two lanes. This may indicate that the reactivity was not affected by the sugars attached to the proteins in the post-translational

modification (Figure 5.7).

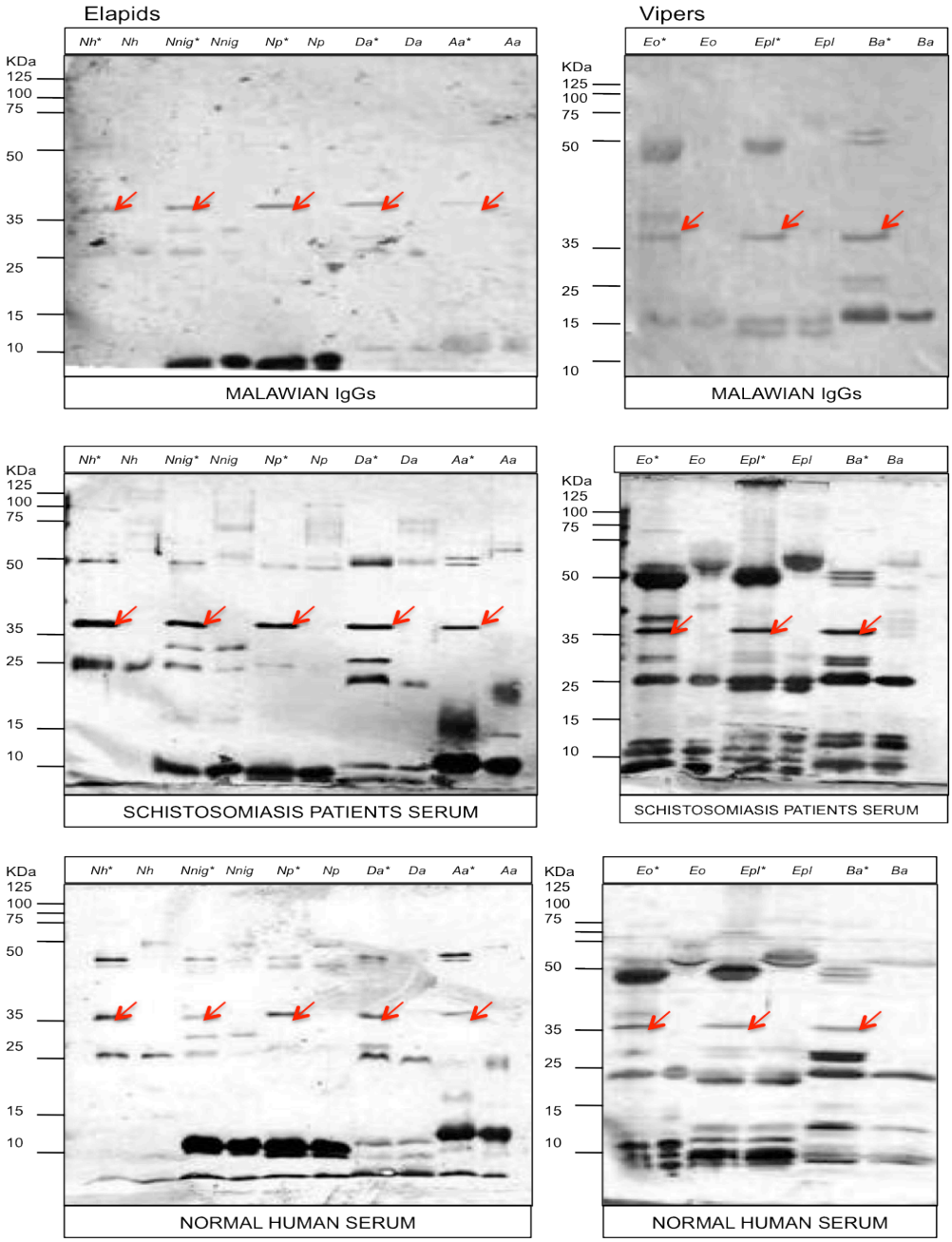


Figure 0.7 Immunoreactivity profile of deglycosylated venom proteins: Immunoblotting profile of eight venoms from the elapids: *N. haje, N. nigricollis, N. pallida, D. angusticeps,* and *A. antarcticus,* and vipers: *E. ocellatus*, *E. p. leakeyi*, *B. arietans*, reactivity toward human sera and immunoglobulin after *in vitro* enzymatic deglycosylation was achieved by the addition of PNGase F (10 units/mL) to 140 µg of venom proteins with an incubation for 1 hour at 37°C. An equal amount of PNGase F treated (*lane) and non-treated (next lane) lysate (10 µg) was loaded and the blots were probed with sera collected from schistosomiasis patients and sera collected from healthy individuals. The molecular weight marker (kDa) is shown on the left of the image. Red arrows indicate the molecular weight of PNGase F at 36 kDa**.**

1.17.3 The titre and avidity of helminth-infected human sera and IVIG preparations to snake venom proteins

To determine the titres of the sera of the helminth-infected human and IVIG interacting with snake venom, and the antigen-binding strength (relative avidity ELISA), an end-point enzyme-linked immunosorbent assay and avidity ELISA were performed. The end-point titre comparisons of the three immunoglobulins and three human sera end-point titres revealed some variation between them.

First, by comparing the results of the IVIG, the IgGs collected from African malaria patients and that collected from normal humans (Malawian and GAMMAGARD), end-point titres were 1:30 and 1:15, respectively (Table 5.1). On the other hand, the IgM (PENTAGARD) showed a higher titre (1:80) than the IgG titres, although it had lower relative avidity (2.05 M) compared with 2.53 and 4.25 M for Malawian IgG and GAMMAGARD (IVIG), respectively. This result might be related to the nature of each immunoglobulin, as IgMs represent the first antibodies to appear in response to the initial exposure to an antigen, and they are not as versatile as IgGs and decline after a short period of infection. The relative avidity shown in the GAMMAGARD (IVIG) (4.25 M) might be explained by the ability of the IgG to bind and neutralise toxins. However, immunoglobulin avidity to venom was not as strong as we were hoping it would be.

Serum samples collected from the patients with filariasis (symptomatic and asymptomatic) exhibited titres against *E. p. leakeyi* venom that varied by about two dilution factors. Interestingly, the schistosomiasis patients' sera collected from non-treated patients demonstrated similar titres to asymptomatic filariasis patients' sera, which were not treated, either. This result agrees with the immunoblotting, as the profiles of the schistosomiasis and asymptomatic filariasis patients showed similar reactivities to snake venom, which might be related to the immune response exhibited by the two parasites. This will be discussed in detail in the Discussion section. The results of the relative avidity ELISA performed on the samples collected from the patients with filariasis and schistosomiasis showed that the relative avidity of the filariasis samples was consistently (but not markedly) more robust than that of the schistosomiasis samples (Table 5.1). This finding did not support our expectations regarding the probability of the presence of natural immunity to venom toxin in patients with parasitic

infections.

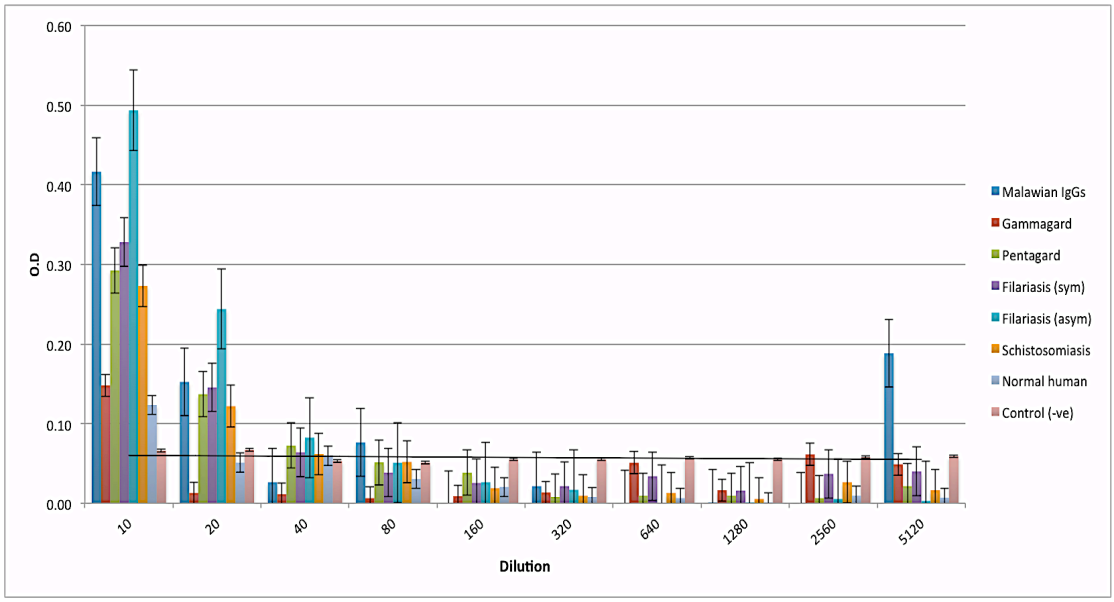


Figure 0.8 End-point titre of helminth-infected human sera and IVIG preparations to snake venom proteins: Bar charts demonstrating the ELISA titres of IVIG preparations (IgGs collected from malaria patients in Malawi and IgGs and IgMs purified from healthy individuals for therapeutic purposes) and sera (collected from symptomatic and asymptomatic filariasis patients, schistosomiasis patients and healthy individuals (Sigma)) were tested against *E. p. leakeyi* venoms. The end-point titre was described as the dilution at which absorbance was greater than that of the negative control (venom only) plus 2 standard deviations (represented by the linear trend line in the graph).

Human serum/ IgG	ELISA	Chaotropic ELISA
	End-point titre	**Relative avidity (M)**
Malawian IgG (malaria patients)	**≈ 3 x 10^{-1}**	**2.53**
GAMMAGARD (human IgG)	**≈ 1.5 x 10^{-1}**	**4.25**
PENTAGARD (human IgM)	**8 x 10^{-1}**	**2.05**
Filariasis (symptomatic) serum	**≈ 6 x 10^{-1}**	**3.02**
Filariasis (asymptomatic) serum	**8 x 10^{-1}**	**3.93**
Schistosomiasis serum	**8 x 10^{-1}**	**0.57**
Normal human serum	**2 x 10^{-1}**	**0.68**

Table 0.1 The end point-titres and relative avidity of immunoglobulins and human sera: three different immunoglobulins (IgGs collected from malaria patients in Malawi, and IgGs and IgMs purified from healthy individuals for therapeutic purposes) and four sera (collected from symptomatic and asymptomatic filariasis patients, schistosomiasis patients and healthy individuals (Sigma)) were tested against *E. p. leakeyi* venoms. Relative avidity is expressed as the amount of ammonium thiocyanate required to reduce the antibody binding to the venom by 50%.

1.18 Discussion

The examination of the presence of immunologic reactivity against snake venom proteins in patients infected with parasitic diseases elicited some interesting observations. First were the obvious differences between asymptomatic and symptomatic filariasis patients. The asymptomatic patients' sera showed higher cross-reactivity ability toward venom proteins in comparison with the symptomatic ones. Interestingly, this immunoreactivity included both high and low molecular weight proteins in viper venom, which represents major toxin proteins in venom like SVMP, LAAO and PLA_2 that play a major role in the pathology caused by viper venom.

When these sera were probed to test elapid venom reactivity, low molecular weight proteins, including PLA_2 and the extremely toxic low immunogenic 3-finger toxins, were the main reacted proteins, the assumption of the proteins were based on Mackessy, 2009. Although this immunoreactivity might be a result of triggering the immune system of the host by parasite proteins similar in function and sharing some homology in the protein sequence with venom proteins, it is interesting to observe such a difference between the two patient groups. This remarkable difference between the two groups might be explained by understanding the role and the effects of filarial nematodes in disturbing and regulating the immune systems of infected patients.

Filariasis patients' immune responses towards the parasite are of the T helper-2 type, which is involved in the production of cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 and the antibody isotypes IgG1, IgG4 and IgE (Babu and Nutman, 2012). It also harbours an expanded population of eosinophils, basophils, mast cells and alternatively activated macrophages. Over time, when patients reach chronic infection, prototypical type-2 responses are modulated by both adaptive and natural regulatory T cells, alternatively activated macrophages, and eosinophils. Filarial infections usually exhibit elevated levels of IgE, which are detected soon after exposure. These IgEs are mostly not antigen-specific, and they persist for many years after treatment for the infection (Mitre and Nutman, 2006). Filarial parasites exert intense immunoregulatory effects on the immune system of the infected host; these effects exhibit both parasite antigen-specific and more generalised levels of immunosuppression (van Riet et al., 2007). Patients with

lymphatic filariasis have shown markedly diminished responses to parasite antigens, as well as some considerable attenuation in response to bystander antigens and routine vaccinations (van Riet et al., 2007). Treating symptomatic patients with the anti-filarial drug ivermectin might also play a role in diverging the immune response of treated patients, and therefore, induce the loss of immunoreactivity to snake venom proteins (Bergquist and Lustigman, 2010). This may explain the differences we observed between blots probed with sera collected from symptomatic patients and asymptomatic ones.

The ELISA results support the previous assumption that the alteration in the immune system caused by the infection triggered the reactivity. In addition, the elevation of the antigen non-specific IgE levels, along with the changes in the immune responses, clarifies the observed low-affinity binding of the human sera to snake venom proteins, which lost their stability with the addition of a low concentration of chaotropes.

The results of the schistosome-infected patients did not show a diverse picture from that of the filarial patients; instead, the reaction sequences showed similar progression. The immunity to the schistosomes resembles the filariasis response in many ways. It is associated with the T helper cell type-2 immune response, which is characterised by elevated levels of IgE, a high production of IL-4, and eosinophilia (Riveau and Capron, 2005). Similar to filariasis, the T regulatory cells and suppressive macrophage populations of schistosome-infected patients might be crucial for down-modulating immune inflammatory responses through infection (Capron et al., 2005). More importantly, schistosomiasis might also have potent systemic immunomodulatory and immunoregulatory effects on the host system towards other infectious and non-infectious antigens (Maizels & Yazdanbakhsh, 2003; Wickelgren, 2004).

The interesting observation in the experiments conducted on the schistosomiasis patients' sera was the difference between results obtained from patients living in low prevalence villages compared with high prevalence villages. The complete loss of immunoreactivity to the high antigenic, low molecular weight elapid venom proteins from blots performed with sera collected from the low prevalence village one year after treatment was surprising. Reactivity toward these venom proteins appeared even in sera collected from healthy individuals. This finding might correlate with the potent systemic immunomodulatory and immunoregulatory effects on the host

system described above (Capron et al., 2005).

Besides the substitutive treatment of patients with primary and secondary antibody deficiencies, therapeutic preparations of normal polyspecific IgG (IVIG) are used in treating a large number of immune-mediated conditions, such as acute and chronic/relapsing autoimmune diseases and systemic inflammatory disorders (Prasad et al., 1998). Furthermore, studies showed that IgGs purified from African populations may have better efficacy when used for treatment than those purified from Europeans, as they have not been persistently stimulated by parasites (Mekhaiel et al., 2011; Rowe et al., 1968). In the present study, we were hoping to discover positive outcomes by immunoreacting snake venom proteins with purified human immunoglobulins.

Malawian IgG showed an interesting pattern of cross-reactivity towards venom proteins similar to that obtained by the sera collected from asymptomatic filariasis patients and non-treated schistosomiasis patients. We were looking for this pattern of immunoreactivity in the Malawian IgG, proposing that these antibodies might have the ability to neutralise some of the venom toxin proteins, similar to those from parasites. This agreed with what the literature was describing about the greater capability of IVIG purified from malaria-infected Africans to neutralise antigens in comparison with IVIGs from European and American donors. Although not expected, the presence of reactivity between low immunogenic proteins in snake venom and the non-specific purified IgGs (GAMMAGARD) may support the above. In contrast, purified IgM (PENTAGARD) showed the expected results, and failed to cross-react with the venom proteins.

Although the IgGs (IVIG and Malawian IgG) showed cross-reactivity towards venom proteins, this reactivity was not robust when tested by ELISA, as the end point titre did not reach higher than 3×10^{-1} in Malawian IgG and 1.5×10^{-1} and 8×10^{-1} in GAMMAGARD and PENTAGARD, respectively. This was supported by the loss of connectivity between the antibodies and the venom when a low concentration of chaotrope was added in the avidity ELISA.

To further investigate if the post-translational modification of the proteins affected the way the venom reacted to human sera and immunoglobulin, a venom protein deglycosylation

experiment was done. The evidence confirms that the immunoreactivity we observed and described in this chapter did not show a significant difference between the deglycosylated proteins and non-deglycosylated ones. Thus, the post-translational modification and the addition of glycan did not affect the reactivity of the venom.

Overall, in going through the information derived from the results described in this chapter, we can conclude that pre-existing parasitic infections generate IgG responses that cross-react, with weak affinity, to snake venom proteins. The low titre and weak affinity of this IgG cross-reactivity precludes us from concluding that parasite-induced IgGs offer any protection to the effects of snake envenoming. Nevertheless, it would be interesting to study sera collected from envenomed patients who are infected or have been infected with parasites, and compare it with non-infected envenomed patients. It would be fascinating to study the differences in pathology and immune reactivity between them.

Chapter 6 The Natural Immunogen Boosting of Vaccination (NIBOVAC)

1.19 Introduction

Despite the effectiveness of antivenom, the mortality and morbidity rates of snakebite remain high in countries (particularly African countries, with 32,000 deaths annually) with the lowest GDP/capita and governmental expenditures on health (Harrison et al. 2009; Kasturiratne et al. 2008; Harrison et al. 2011). The high cost (>$100/vial) and logistical difficulties of delivery explain the poor demand for antivenom in these countries (Harrison et al. 2009). Research encouragement to broaden the geographic efficacy and improve the commercial viability of antivenom therapy is compelled by several factors. There are promising developments in experimental tests in this area, involving 'antivenomics'(Wagstaff et al. 2009; Lomonte et al. 2008; Calvete et al. 2010; Calvete et al. 2009) and 'epitope-string immunogen' (Harrison 2004; Wagstaff et al. 2006) approaches, that aim to amplify the clinical and snake-species efficacy of snakebite serotherapy and to reduce manufacturing costs. On the other hand, several studies in the early 50s have tested the efficiency of vaccination against snakebite (Sawai et al. 1969; Someya et al. 1972). They represented a promising neutralization of toxic effects of snake venom. However, the developed immunity did not persist for long periods as envenomation occurred in immunized individuals when bitten 2-years post immunization (Flowers 1963).

The study of epidemiological maps indicates that populations residing in geographic zones with a high risk of snakebite also suffer from the medical burden of vector-transmitted diseases and neglected tropical diseases (NTD) (Harrison et al. 2009; Harrison et al. 2011). Disease vectors, such as mosquitoes, coincide with snakebites during rainfall season. The probability of high snakebite incidence is increased at the beginning of the rainy season (Kasturiratne et al. 2008). Rainfall facilitates the growth of mosquito populations, resulting in an overlapping period during the rainy season where both snakebite incidence and the number of mosquitoes increase (Chaves and Kitron 2011; Chaves et al. 2011).

All of the aforementioned factors, in addition to, the fact that some mosquitoes salivary proteins are of the same protein types as some of the most pathogenic venom proteins – metalloproteinases (Francischetti 2010; Arcà et al. 2005) led to the development of the Natural Immunogen Boosting of Vaccination (NIBOVAC) vaccine concept by Harrison of the Alistair Reid Venom Research Unit. NIBOVAC offers an alternative, mass-treatment, and thus cost-

effective approach using natural boosting through mosquito bites to generate high titres of circulating anti-toxin IgG to neutralise envenoming.

NIBOVAC was conceptualised as a potential prophylactic therapy for snakebite on the basis that: (i) immunity to the lethal effects of snakebite has developed in horses hyper-immunised with venom during antivenom production, (ii) the capability of IgGs specific to discrete venom toxin motifs to neutralise venom-induced lethality (Wagstaff et al. 2006; Iddon et al. 1988) and (iii) the coincidence between West Africa *E. ocellatus* viper bites and mosquito bites during the rainy season (Molesworth et al. 2003). Concerning NIBOVAC feasibility, the most critical challenge is determining whether a primary immunogen can be designed such that the seasonal surge in mosquito bites (which provoke immunogenic responses in both endemic and temporal human populations (Fontaine et al. 2011; Orlandi-Pradines et al. 2007; Titus et al. 2006) can be manipulated to boost circulating anti-toxin IgGs naturally at the time of peak snakebite risk. Conventional vaccination against snakebite is impractical because the lethal effects occur rapidly (6–24 hours post-bite) and well before protection could be mounted by a recalled immunological response (Müller et al. 2012).

NIBOVAC is completely a novel vaccine perception designed to exploit natural immunogens to stimulate sustained levels of protective immunity against snakebite. The success of NIBOVAC will (i) preclude the current requirement for conventional boosting vaccination, which is considered one of the obstacles in vaccination against snakebite and constitutes a financial and logistic impediment to the uptake and effectiveness of many vaccine programs and (ii) establish pre-existing protective immunity for the rapid onset of snakebite pathology. One of the barriers to vaccination against snakebite is that conventional vaccination establishes dormant immune memory, which is activated into protective immunity by antigens from venom. Therefore, there is a several-day delay before the ‘vaccinee’ is protected from pathology/death by the immune response, which prevents the implementation of vaccine for snakebite, as pathological manifestations are very rapid (Chippaux and Goyffon 1998).

To attain our goal, we selected mosquito bites (day and night biters; natural booster) and haemorrhagic venom from West Africa, *E. ocellatus*, as test models. We utilised a ‘reverse vaccinology’ approach (Donati and Rappuoli 2013) to detect multiple epitopes for each toxin/mosquito salivary protein (MSP; sialome) family that is predicted to be immunologically

cross-reactive. Multiple linear B-cell and T-cell epitopes, designed to direct IgG to the native epitope, identified by the computational selection of short, conserved peptide sequences predicted to be surface-exposed in toxin/MSP macromolecular structures. The identified epitopes subjected to an amino acid sequence manipulation technique used at the Alistair Reid venom research unit, to devise 6–12 overlapping AA peptides, a set of chimeric epitope candidate immunogens best representing the conformation of the native epitopes (Wagstaff et al. 2006). The designed epitopes will be conjugated to carbon nanotubes (phase 1). In phase 2, with local Ethics Committee approval, three groups of 10 mice vaccinated once in week 0, 4 or 8 with NIBOVAC-carbon nanotube immunogen. Then, in week 12, the mice are subjected to two weeks (three times/week) of mosquito feeding. In week 16, the mouse groups subjected to assays approved by the WHO to determine the IgG neutralisation of venom-induced haemorrhage whereby the mice intradermally injected with venom followed by examination of the haemorrhagic lesion size 24 hours post-injection. Throughout the experiment, sera samples collected and examined for the temporal induction of IgG specific to venom toxin epitopes predicted from heir sequences. Control groups included: mice receiving (i) only mosquito bites and (ii) only the NIBOVAC immunogen. The measurement of hypersensitivity induction to MSP in all groups is undertaken by measuring MSP-specific IgE. This chapter will discuss the methods and the results of phase one of this study that include epitope selection, design and construction, as the time elected for this PhD restricted this study to phase one. Phase two that will examine the feasibility of NIBOVAC concept by vaccinating mice with the peptide immunogens conjugated to carbon nanotubes followed by assessing specificity, titre, avidity and venom-neutralising efficacy of IgG from vaccinated mice. And finally validate the NIBOVAC concept in vaccinated mice subjected to mosquito-feeding and then challenged with snake venom will be conducted in an alternative study.

1.20 Materials and Methods

1.20.1 Snake venom and mosquito sialome database construction

After searching mosquito sialome databases and studying related literature, databases were constructed for each protein group from mosquito sialomes (day and night biters) that have a parallel in snake venom toxin proteins (Holt et al. 2002; Valenzuela et al. 2002; Francischetti et

al. 2002; Valenzuela et al. 2003; Ribeiro et al. 2004; Kalume et al. 2005). Of these, databases populated with sequences, of publicly available mosquito sialome database or a published paper, representing each protein group: mosquito sialome metalloprotease database (db-MSPMP), serine protease (db-MSPSP), phospholipase A_2 (db-MSPPLA_2) and c-type lectin (db-MSPCLP) were constructed. The sequences from each database were organised according to mosquito species represented by the sequence *An. gambiae, Ae. aegypti* and *Cu. quinquefasciatus.* Parallel databases for snake venom toxin (*E. ocellatus*) (Wagstaff and Harrison 2006) proteins were constructed: db-SVMP, db-SVSP, db-SVPLA_2 and db-SVCLP.

1.20.2 Bioinformatics

A homology search between snake venom databases and MSP was undertaken using an Intel, dual 2.8GHz RedHat Enterprise Linux 9.0 workstation running Biolinux 6.0 (available at http://envgen.nox.ac.uk). Sequences were analysed using blastp (protein-protein BLAST) protein sequences searching program, and the top five hit sequences were selected from each database. The MSP databases were aligned to parallels from snake venom databases using the CLUSTAL alignment tool in Jalview a free program for multiple sequence alignment editing, visualization and analysis (available at; http://www.jalview.org)(Waterhouse et al. 2009).

1.20.3 Electrophoresis analysis and immunoblotting

The source and preparation of the samples are described in detail in Chapter 2, Section 2.2. The blotting protocol and the antivenom/toxin-specific IgGs were used as described in Chapter 2, Section 2.5 and Chapter 4, Section 4.3.1.

1.20.4 Chimeric epitope construction

To predict epitopes of homologous snake venom (*E. ocellatus*) and MSP as primary vaccines that are immunologically cross-reactive, a reverse vaccinology approach utilised. After identifying MSP sequences that are homologous to snake venom toxins from each protein group, the FASTA format files containing homologous MSP and snake venom sequences, divided into groups according to protein type, similar to the databases used previously, were loaded on BepiPred to predict multiple linear B-cell epitopes designed to direct IgG to the native epitope (available at http://www.cbs.dtu.dk/services/BepiPred/) (Larsen et al. 2006). In parallel, CD4+ T-cell epitopes predicted to bind with high affinity to the human MHC class II cell surface

receptors of HLA-DR1 alleles were simultaneously identified through a similar approach using NetMHCIIpan-2.0 (available at http://www.cbs.dtu.dk/services/NetMHCIIpan-2.0/) (Nielsen et al. 2010). This specific allele was selected for this model system because of its wide expression in human populations, including West African populations, and the availability of HLA-DR1 transgenic mice (Hill et al. 1992; Richards et al. 2009) that will permit testing of the concept.

The linearity of the predicted epitopes was tested using ElliPro (available at http://tools.immuneepitope.org/tools/ElliPro/iedb_input) (Ponomarenko et al. 2008). The protein sequences in FASTA file format were loaded to the server and the default parameters were used in the search. The parameters used in the application predicted the structural templates from PDB that used to model a 3D structure of the submitted sequence.

1.20.5 Prediction of antigenicity and surface exposure of predicted epitopes

The Protean Software (Lasergene software, DNASTAR, Madison, Wisconsin, USA) was used for protein structure analysis and prediction to locate the most antigenic domains and surface probability for each previously predicted epitope sequence. Following the identification of the most antigenic epitopes, their surface exposure was confirmed by overlay using YASARA program (Yet Another Scientific Artificial Reality Application) (http://www.yasara.org) on available models of snake venom protein (3DSL; Bothropasin a PIII snake venom metalloprotease, 2AIP; serine protease protein C activator, 1OZ7; Echicetin c-type lectin from *E. carinatus*) (Muniz et al. 2008; Jasti et al. 2004; Murakami & Arni 2005). These crystallographic data were selected using the ConSurf server (http://consurf.tau.ac.il) (Landau et al. 2005). The alignments of the snake venom and MSP protein sequences were loaded on the server, and the closest 3D structure was selected.

1.21 Results

1.21.1 Identification of pathogenic venom toxin group homologues in mosquito sialomes

Preliminary to the epitope design, I identified homologues of each pathogenic venom toxin group (metalloproteinases, phospholipase A_2s, serine proteases and C-type lectins) in mosquito sialome datasets. The databases were populated by assembled contigs of *E. ocellatus* venom toxin proteins and blasted with the MSP databases populated with selected mosquito sialomes protein sequences belonging to proteases found in both mosquitoes and snake venom in available literature.

Databases BLAST search was undertaken on an Intel, dual 2.8GHz RedHat Enterprise Linux 9.0 workstation running Biolinux 6.0 (available at http://envgen.nox.ac.uk). The inferred homology of the full-length comparative sequence analysis of the snake venom protein and mosquito sialomes, though of low identity percentage (around 30%, provided in Figure 6.1), illustrates the degree of sequence and domain conservation. First, metalloproteases from mosquito sialomes exhibited the conserved $\alpha_2\beta_1$ ECD motif known to be part of the disintegrin-like domain in SVMP (Fox and Serrano, 2005; Wagstaff and Harrison, 2006). Additionally, the alignment of metalloproteases identified the DLPEY motif in mosquitoes and SVMP transcripts, which have been found to be conserved in ADAM family members that bind $\alpha_9\beta_1$ integrin (Eto et al., 2002; Wagstaff and Harrison, 2006). The sequences of SVMP and mosquito sialomes not only showed conservation in only these two motifs; several other motifs were identified as well (Figure 6.1-a).

Serine proteases also showed homology in both SVSP and sialomes. Conservation surrounding some serine proteinase evolutionary markers and active site residues for the S1 family of clan SA were detected. These motifs are TAAHC, surrounding the catalytic His, and GDSGGP around Ser (Figure 6.1-b) (Serrano and Maroun, 2005).

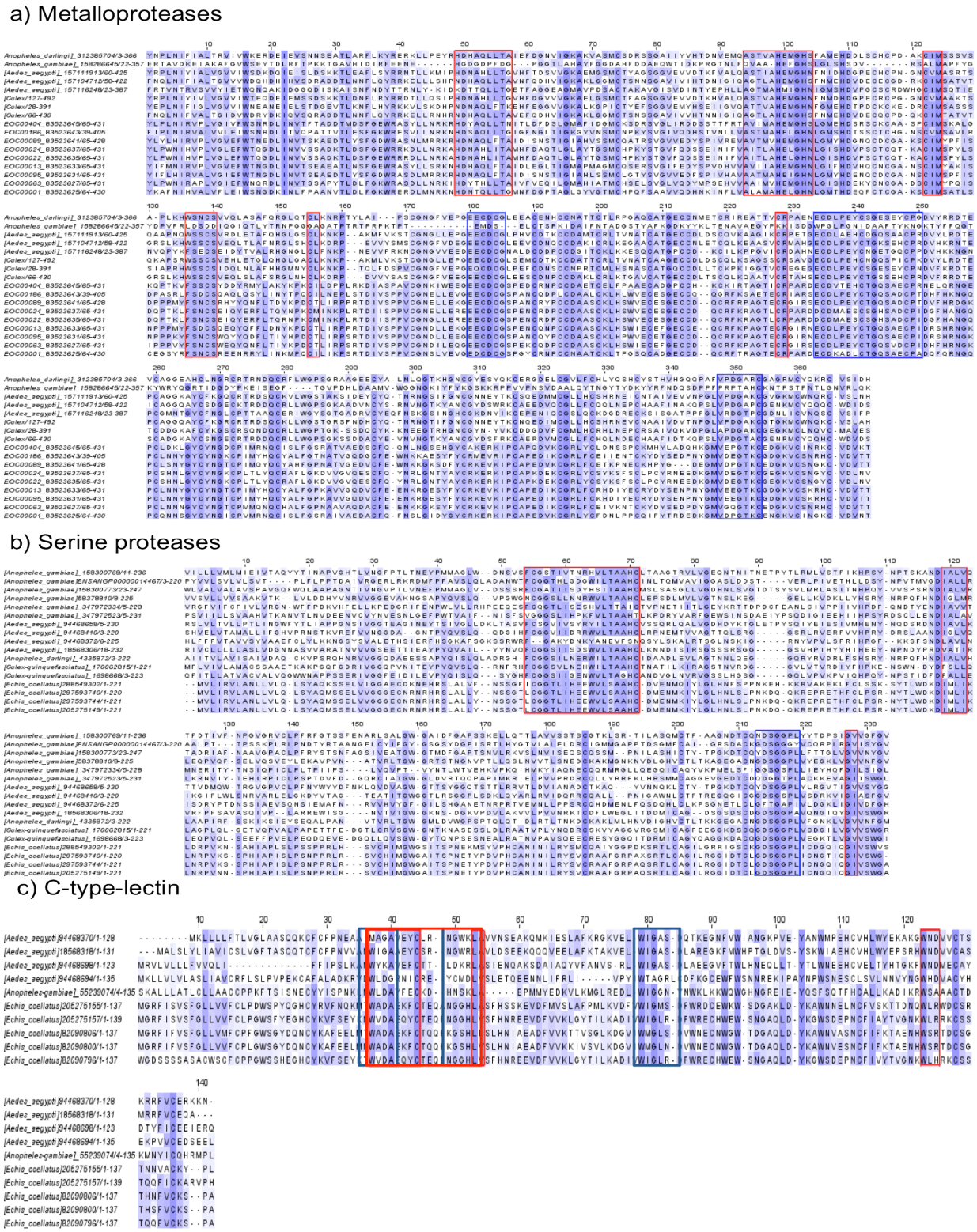


Figure 6.1 Snake venom proteins and MSP alignment: sequence alignment using Jalview of venom and mosquito a) metalloprotease, b) serine protease and c) c-type lectin. Blue and red boxes indicate preliminary T- & B-cell epitopes identified by bioinformatics analysis.

Similarly, c-type lectin homology was found to be within the carbohydrate recognition domain (CRD). C-type lectin proteins containing this domain bind to γ-carboxyglutamic acid (Gla)-containing domains of coagulation factors IX and X in the presence of calcium ions (Figure 6.1-c) (Morita 2004; Arlinghaus and Eble 2012; Ogawa et al. 2005).

1.21.2 Reactivity of snake antivenoms and toxin-specific IgGs to mosquito salivary gland proteins (MSP)

i) Antivenoms (monospecific and polyspecific)

The goal of this study was to corroborate the above bioinformatics predictions by investigating whether antivenom IgGs bind to proteins in mosquito saliva (MSP) and, if so, determine whether the MSPs were indeed similar to pathogenic venom proteins. The antivenoms used in these experiments were selected according to the same criteria used in Chapter 4, as they are all active against African snakes.

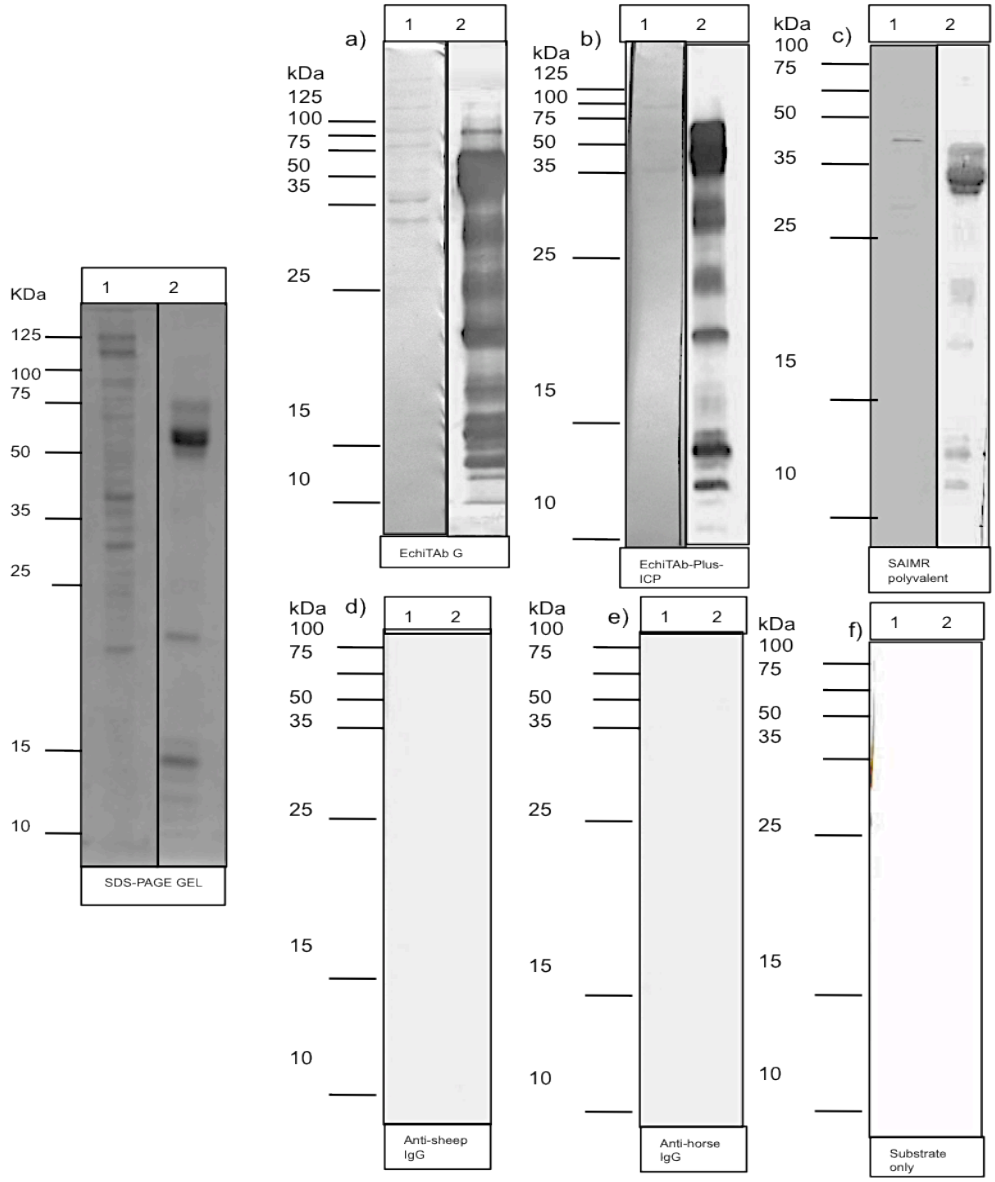


Figure 6.2 Protein profiling and immunoblotting: reduced 15% SDS-PAGE gel Coomassie blue stained and immunoblotts against various antivenoms: African a) monospecific EchiTAb G, b) polyspecific EchiTAb-Plus-ICP and c) SAIMR polyvalent. As controls: secondary antibody d) donkey anti-sheep IgG, e) rabbit anti-horse IgG and f) substrate only. 5µg of, non-blood fed, female *An. gambiae* salivary gland homogenate (lane 1) and *E. ocellatus* venom (lane 2) were loaded. The molecular weight markers (kDa) are shown on the left side of the image.

Antivenoms provide immunological cross-reactivity to MSP. As Figure 6.2 illustrates, several proteins exhibited reactivity to EchiTAbG, the *E. ocellatus* monospecific antivenom. The reaction showed specificity to proteins mainly in the high molecular mass range of 30 to around

200 kDa molecular mass. This range correlates with the following MSP proteins: the D7 related protein 33.4kDa, the salivary gland 5 protein 35.8kDa, the serine protease 40kDa, the salivary gland 1-like2 protein 41.4kDa, the salivary gland 1 44.2kDa, calerticulin 44.6kDa, metalloprotease 50.2kDa and 5'-nucleotidase 61.1kDa (Ribeiro et al. 2004; Francischetti et al. 2002; Gorman et al. 2000; Valenzuela et al. 2002; Alvarez et al. 2012). Moving from the monospecific to the polyspecific, the number of reacted proteins decreases. However, the opposite was expected, we used monospecific and polyspecific antivenoms as polyspecific antivenoms have antibodies to venom toxin from more than one single species or even more than one family (vipers and elapids). However, the interesting result is that, in the correlation between the blotting results and molecular weights from studies done on MSP includes proteins, we detected homology in their protein sequences and venom toxin proteins (i.e. metalloprotease and serine protease).

ii) Toxin-specific IgGs

To be more specific and to eliminate any cross-reactivity to proteins other than those that are homologous to venom toxin, toxin-specific IgGs immunoblotting was done (Figure 6.3). Blots were probed with IgGs raised by the Alistair Reid Venom Research Unit to SVMP, SVSP and c-type lectins. For SVMP, IgGs developed against different subclasses were used: the anti-SVMP PI, anti-SVMP PII and anti-disentigrin domains. Figure 6.3 illustrates that probing MSP to SVMPI (Figure 6.3-1) showed restricted specificity to proteins, mainly in the 75 to 100 kDa molecular mass range, although weaker cross-reactivity was also detected at lower molecular weights. In contrast, anti-SVMP PII showed weak cross-reactivity to all proteins ranging between the molecular weights of 35 and 100 kDa (Figure 6.3-2). Anti-disintegrin, an IgG developed against the disintegrin domain, a polypeptide that interacts with integrins in cell membranes and is known to be present in snake venoms, showed intense, restricted specificity to MSP at a molecular weight of ~90 kDa (Figure 6.3-3). Comparing these results with the molecular weights of toxin proteins that these IgGs were prepared to neutralise, we can observe that some are in accord with those of venom protein and MSP, while others have a very distinct molecular weight. For example, despite the intense reactivity to SVMP PI, all the proteins that reacted appeared in the high molecular weight range, while the molecular weight of SVMP, that this specific IgG was prepared to, have a lower molecular weight (~21 kDa) (MacKessy 2009).

Similarly, anti-disintegrin and anti-c-type lectin IgGs showed reactivity to high molecular weight MSP proteins, while they are of a low molecular weight (disintegrin in snake venom around 10 kDa and c-type lectin in both snake venom and MSP of around 14 kDa) (Valenzuela et al. 2002; MacKessy 2009). Another factor that can be noted here is the presence of cross-reactivity between MSP and snake venom disintegrin. Anti-disintegrin IgG was developed against snake venom disintegrin and anti-integrin molecules found in viper venom (Marcinkiewicz 2005; Juárez et al. 2008). This detection of anti-disintegrin IgG to an MSP protein with high specificity despite the fact that the molecular weight is different from that of snake venom disintegrin, is interesting. Furthermore, the close molecular weight of the protein detected by anti-c-type lectin to that detected by anti-disintigrin is of questioning whether this mosquito protein has the ability to bind to host integrin similar to snake venom disintegrin and c-type lectin (Marcinkiewicz 2005; Ogawa et al. 2005).

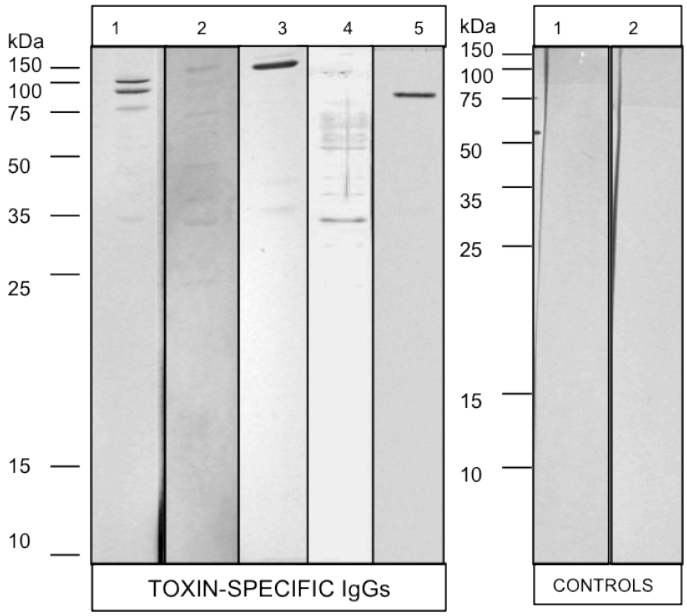


Figure 6.3 Toxin specific-IgG reactivity: reduced immunoblotting (15% SDS-PAGE gel) against various toxin-specific IgGs and controls: snake venom metalloprotease (1) ANTI-SVMP- P I IgG, 2) ANTI-SVMP-PII IgG and 3) ANTI-DIS); serine protease SP (4) ANTI-SP); c-type lectin (5) ANTI_CTL I). Controls; 1) normal mouse serum, 2) goat anti-mouse IgG tested against 5µg of *An. gambiae* salivary gland homogenate. The molecular weight markers (kDa) are shown on the left side of the image.

On the other hand, anti-SVMP PII and anti-SP cross-reacted to diverse MSPs in wide molecular mass ranges of 35–150 kDa, which includes the weights of these proteins in both snake venom and MSP (Figures 6.3-2, 4). This might reflect the observed homology in protein sequences and the present conserved sites between snake venom and MSP metalloproteases and serine proteases.

1.21.3 Epitope construction

The preliminary data obtained from bioinformatics and immunoblotting indicates that there exist domains within some proteins groups that are shared between MSPs and pathogenic snake venom proteins. Our intent is to manipulate these domains to produce chimeric immunising peptides representing both venom toxin and MSP. For this purpose, a *in silico* vaccine predection approach to identify multiple epitopes for each toxin/MSP family that is predicted to be immunologically cross-reactive was performed (Wagstaff et al. 2006). Multiple linear B-cell epitopes, designed to direct IgG to the native epitope, were identified by computational selection. These epitopes are of short, conserved peptide sequences (5–7aa) predicted to be surface-exposed in toxin/MSP macromolecular structures. In addition, CD4+ T-cell epitopes (12–15aa), predicted to bind with high affinity to the human MHC class II cell surface receptors of HLA-DR1 alleles, were identified in a similar manner.

After the alignment of protein sequences from *E. ocellatus* venom proteins and MSP, the prediction of B-cell and T-cell epitopes was done according to the criteria described above. The prediction was done using BepiPred, a Web tool that predicts B-cell epitopes (available at http://www.cbs.dtu.dk/services/BepiPred/) (Larsen et al. 2006) in accordance with NetMHCIIpan-2.0 (available at http://www.cbs.dtu.dk/services/NetMHCIIpan-2.0/) (Nielsen et al. 2010) to predict the relative binding strengths of all possible nonapeptides to MHC class II molecule HLA-DR1 (available at http://www-bimas.cit.nih.gov/molbio/hla_bind/) (Parker et al. 1994). Additionally, to detect the linearity of these epitopes, the ElliPro Web tool was used (http://tools.immuneepitope.org/tools/ElliPro/iedb_input) (Ponomarenko et al. 2008).

Peptide prediction was conducted through an analysis of MSP and snake venom protein

databases in addition to the available related 3D structure of the protein using the aforementioned Web tools. This analysis identified seven domains whose antigenic profiles are similarly conserved across SVMP isoforms and homologues from MSP, two domains from serine protease and three from c-type lectin (Figures 6.4-5–7). These antigenic domains were aligned and colour-identified.

Following epitope prediction, the selected epitopes were scored for the degree of antigenicity and surface exposure probability using the Protean software (DNASTAR, Madison, Wisconsin, United States). In addition, surface exposure was confirmed by overlay using YSARA (http://www.yasara.org) on available models of snake venom protein (3DSL; Bothropasin a PIII snake venom metalloprotease, 2AIP; serine protease protein C activator, 1OZ7; Echicetin c-type lectin from *E. carinatus*) (Muniz et al. 2008; Jasti et al. 2004; Murakami & Arni 2005). These crystallographic data was selected using the ConSurf server (http://consurf.tau.ac.il) (Landau et al. 2005). The alignments of snake venom and MSP protein sequences were loaded on the server, and the closest 3D structure was selected.

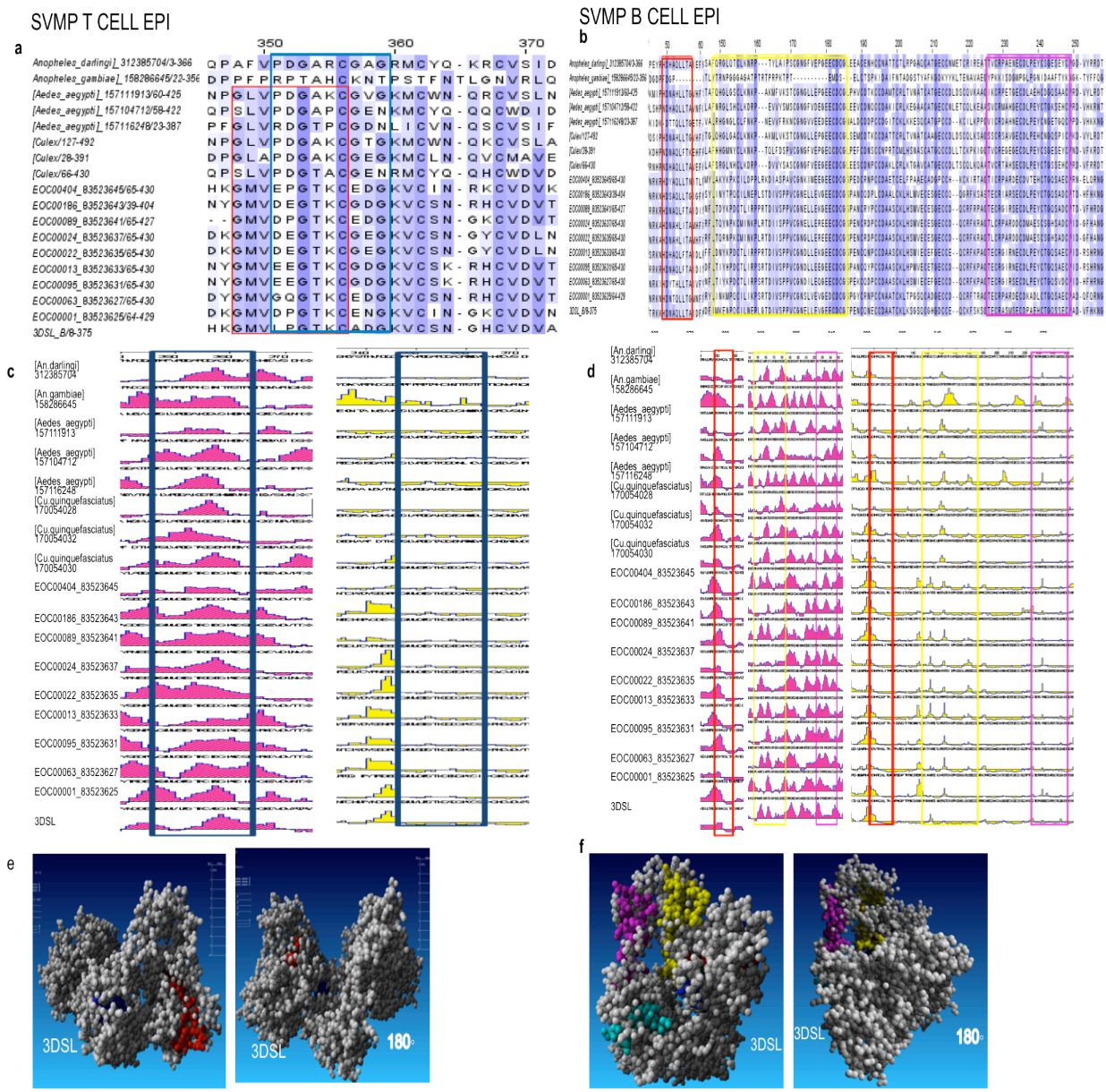


Figure 6.4 Metalloprotease T- & B-cell epitope prediction: metalloprotease venom toxin and mosquito protein homologues. Percentage identity sequence alignments display identified T (a) and B (b) cell epitopes identified by bioinformatics analysis and the prediction of antigenicity and surface exposure (c, d). Mapping of B- & T-cell epitopes to the macromolecular structure (PDB: 3DSL) (e, f) with the greatest similarity to mosquito and venom metalloprotease, rotated 180°. Note that the metalloprotease alignment is not full-length; the displayed alignment represents the T- & B-cell epitope with high antigenicity. Additional epitopes (not displayed) were identified in other regions of the protein but showed lower antigenicity.

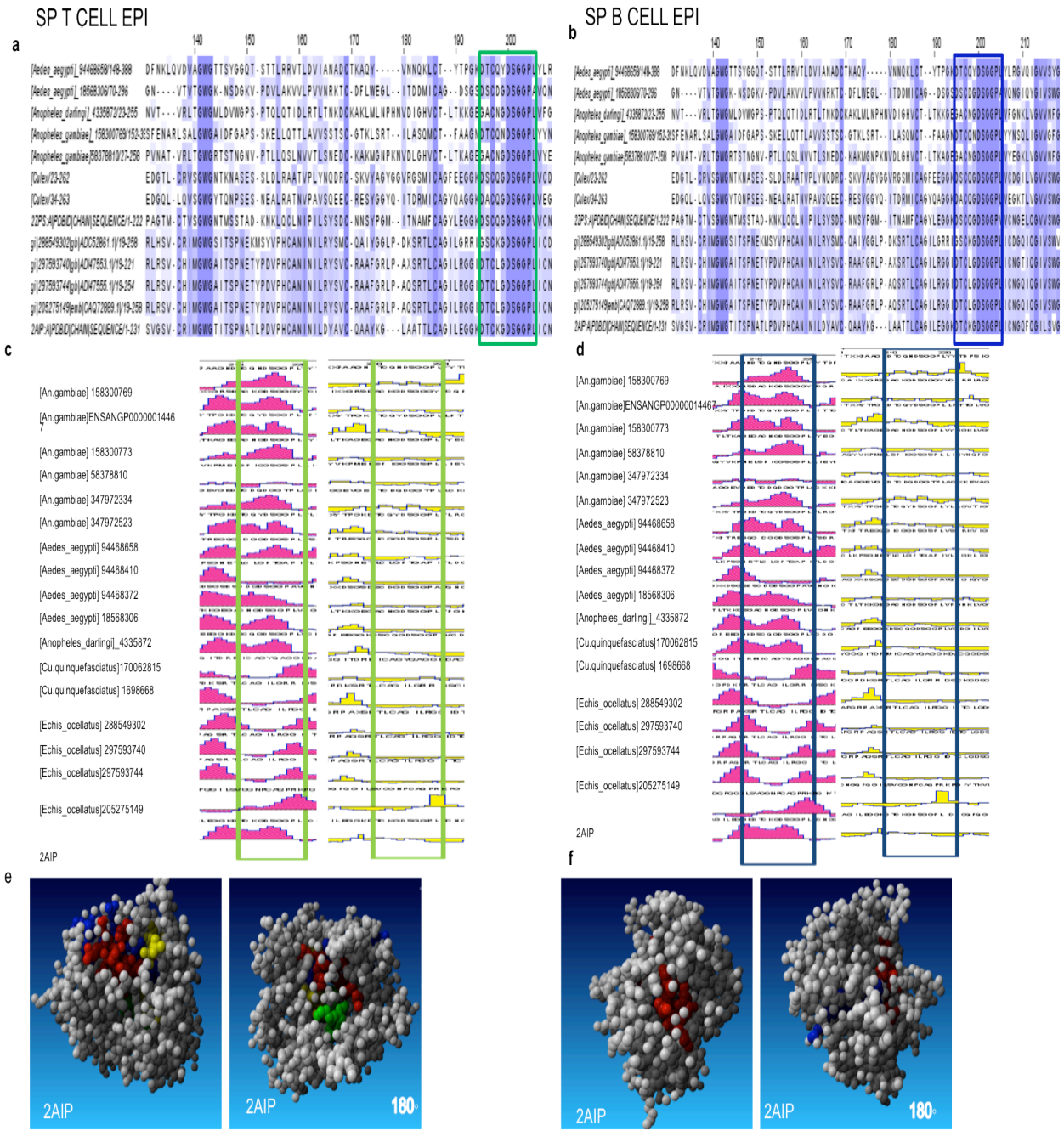


Figure 6.5 Serine protease B- & T-cell epitope prediction: serine protease venom toxin and mosquito protein homologues. Percentage identity sequence alignments display identified T (a) and B (b) cell epitopes identified by bioinformatics analysis and prediction of antigenicity and surface exposure (c, d). Mapping of B- & T-cell epitopes to the macromolecular structure (PDB: 2AIP) (e, f) with the greatest similarity to mosquito and venom serine protease, rotated 180°. Note that the serine protease alignment is not full-length; the displayed alignment represents the T- & B-cell epitope with high antigenicity. Additional epitopes (not displayed) were identified in other regions of the protein but showed lower antigenicity.

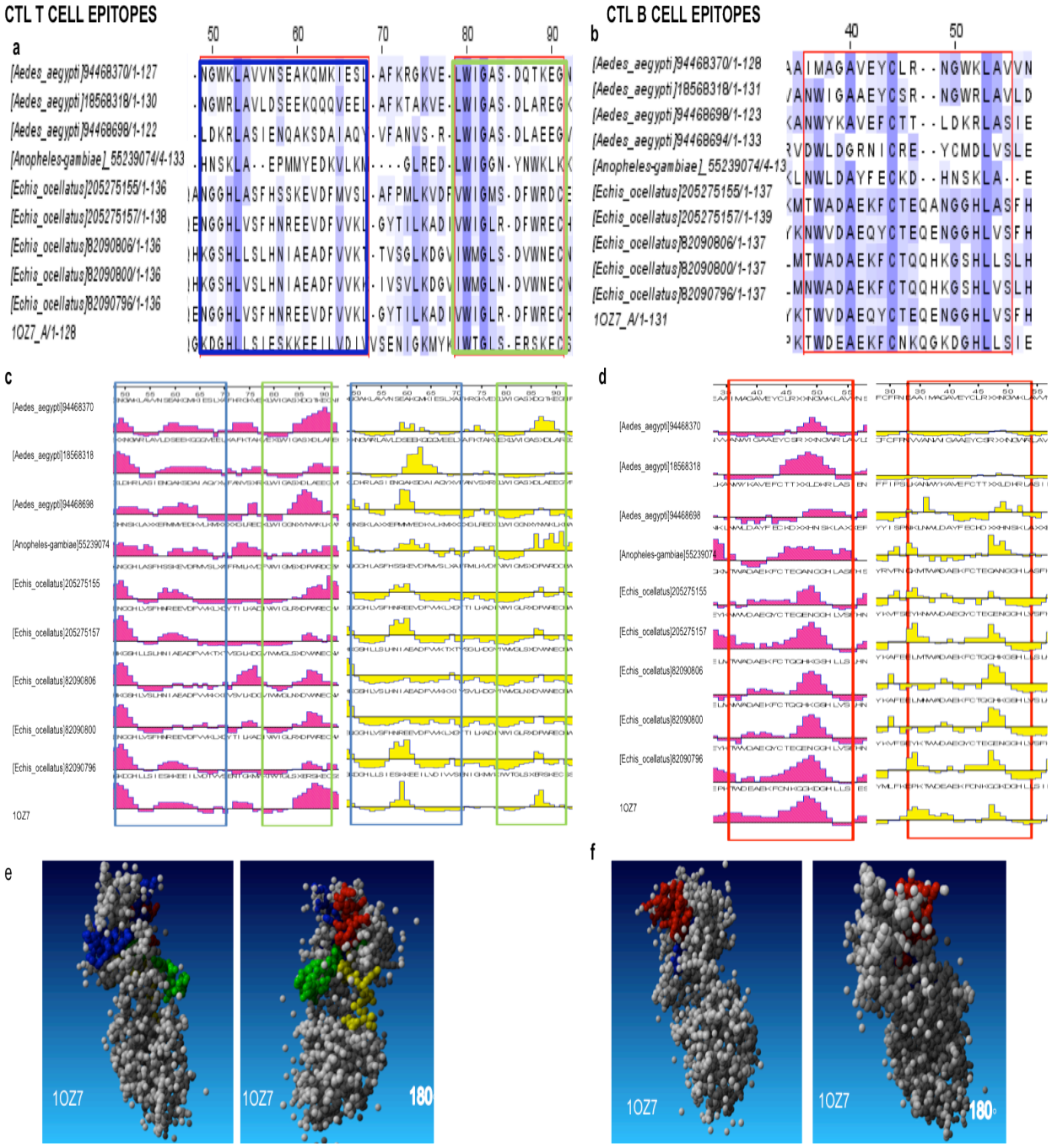


Figure 6.6 C-type lectin T- & B-cell epitope prediction: c-type lectin venom toxin and mosquito protein homologues. Percentage identity sequence alignments display identified T (a) and B (b) cell epitopes identified by bioinformatics analysis and prediction of antigenicity and surface exposure (c, d). Mapping of T- & B-cell epitopes to the macromolecular structure (PDB: 1OZ7) (e, f) with the greatest similarity to mosquito and venom c-type lectin, rotated 180°. Note that the c-type lectin alignment is not full-length; the displayed alignment represents the T- & B-cell epitope with high antigenicity. Additional epitopes (not displayed) were identified in other regions of the protein but showed lower antigenicity.

Following extraction, antigenic profiling and alignment of selected peptides, a second, custom-designed data mining solution (db-2) (a database created at the Alistair Reid venom research unit) scored epitopes for cross-reactivity against all available Echis ESTs and identified small antigenic peptides with broad predicted toxin-neutralising potential. In addition, the length of each antigenic peptide was incrementally reduced and the peptide composition refined into 6-12 amino acids that represented the maximum number of matches to venom proteins clusters and ESTs.

Peptide	# of hits in venom	Anopheles	Culex
IWSNGD	**59**	**WNTRDNIWSNGD**	**-**
IWSNGDK	**22**	**WNTRDIWSNGDK**	**-**
SNRDL	**16**	**-**	**DRYDSNRDL**
NQRDL	**13**	**-**	**DRYDNQRDL**
TQGDL	**9**	**-**	**DRYDTQGDL**
FWNQRDL	**8**	**WNTRDFWNQRDL**	**-**
FWNQR	**8**	**WNTRDNFWNQR**	**-**
HDGNQ	**87**	**TEECSHDGNQ**	**HDTPDCKHDGNQ HDVDGECHDGNQ**
HDGNQC	**87**	**-**	**HDTPDCHDGNQC HDVDGHDGNQC**
CGANS	**39**	**HDTEECSCGANS**	**HDTPDCKCKCGANS**
CGAKS	**23**	**HDTEECSCGAKS**	**HDTPDCKCKCGAKS**
CGADS	**15**	**HDTEECSCGADS**	**HDTPDCKCKCGADS HDVDGECQCGADS**
HDVPS	**14**	**TEECSHDVPS**	**-**

Table 6.1 Total number of individual venom ESTs matched to each venom part of the chimeric peptide predicted using db-2.

Then, the epitope regions selected *in silico* were synthesised into chimeric epitopes by constructing overlapping peptide libraries; each peptide contains part of a snake venom protein and MSP. The length of each peptide was 12 amino acids. Following peptide library construction, the new peptides were again tested for antigenicity. This analysis identified 13 potential epitopes that represented the maximum number of matches to SVMP clusters and ESTs and showed a high antigenicity profile (Table 6.1). Peptides identified from SP and c-type lectin had weaker antigenicity or were buried within the protein in comparison with those from SVMP and therefore were excluded.

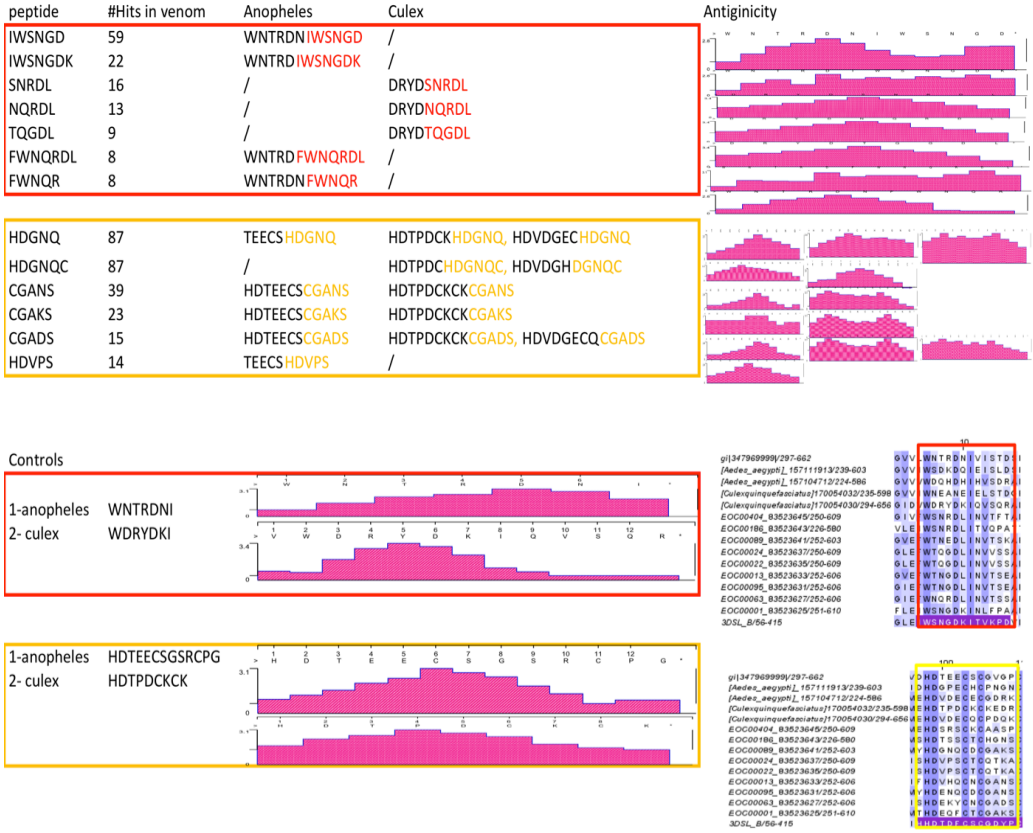
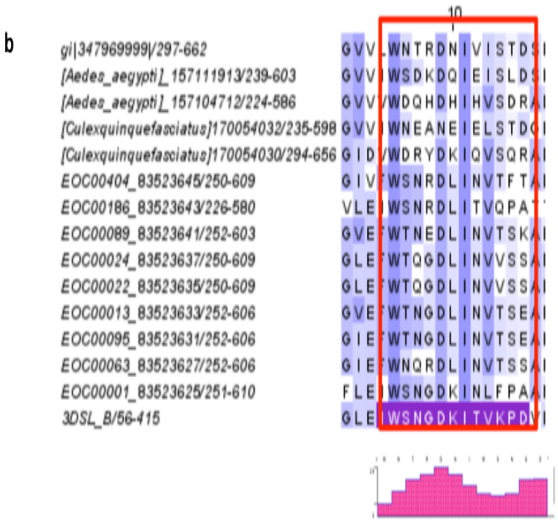


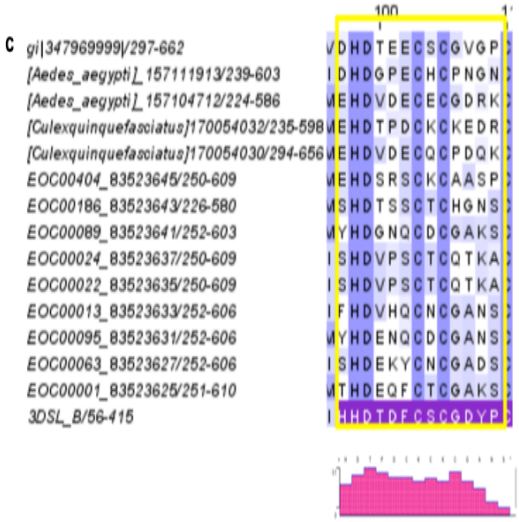
Figure 6.7 Epitopes predicted from metalloprotease: red box (SVMP II), yellow box (SVMP III), first column represent individual metalloprotease epitopes identified from conserved domains between venom and MSP. The number of venom ESTs matched against each peptide is shown in column 2. Columns 3 and 4 represent the designed chimeric epitope, amino acids in black represent the MSP amino acids identifed in the conserved domain between MSP and snake venom metalloprotease (from *An. gambiae* and *Cu. Quinquefasciatus*) followed by venom amino acids from the consarved domain (red represent (SVMP II), yellow represent (SVMP III). Upper right graphs: Jameson-Wolf antigenic profiling of the constructed chimeric epitopes. Lower left graphs: Jameson-Wolf antigenic profiling of the selected controls only from MSP peptides. Lower right: percentage identity alignment of target domains for individual MP epitopes.

The metalloprotease peptides represented two domains, SVMP PII and SVMP PIII (Figure 6.7). Therefore, the epitopes with the highest number of hits in the Echis venom EST data (db-2) was selected from each group. In addition, two control epitopes was constructed each one selected from predicted epitopes representing snake venom only, from each SVMP (II and III) domain, as a positive control. Another two controls were constructed as scrambled epitopes with no antigenicity as a negative control using the MIMOTOPES Web tool (http://www.mimotopes.com/peptidelibraryscreening.asp?id=97). The selected epitopes were then sent to Dr Steven L. Cobb's lab at Durham University for epitope manufacturing and conjugation to carbon nanotubes (Figure 6.8).

a Immunogens

peptide	#Hits in venom	Anopheles	Culex	Antiginicity
IWSNGD	59	WNTRDNIWSNGD	/	
CGANS	39	HDTEECSCGANS	HDTPDCKCGANS	





d Controls

1-anopheles	WNTRDNIVISTD
2- culex	HDTPDCKCKEDR
3-SVMP MET2	WNQRDLINVT
4-SVMP MET3	HDENQCDGANS
5-Scrambled peptide control 1	DSDTVINNWITR
6-Scrambled peptide control 2	CDDTKEHCRKPD

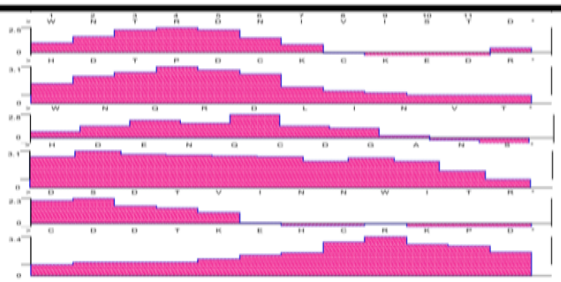


Figure 6.8 Epitopes sent for manufacturing and conjugation to carbon nanotubes: a) immunogens represent chimeric epitopes (red represents SVMP II; yellow represents SVMP III), b) Jameson-Wolf antigenic profiling of the conserved domain representing each immunogen group (venom and MSP). c) Controls sent along with the epitopes; the first two MSPs were selected from the conserved domain; the following two venom only 3-SVMPII 4- SVMP III represent the same conserved domains the MSP controls selected from, and known to induce immunological cross-reactivity to *E. ocellatus* venom (unpublished work by Camila Renjifo at the Alistair Reid venom research unit) as a positive control. 5 and 6 are scrambled epitopes as negative controls.

1.22 Discussion

The genomic/transcriptomic/proteomic data available for snake venom proteins and MSP provides wealthy resources for comparative protein composition. This establishes a critical supply for bioinformatically designed cross-conserved chimeric epitope immunogens. Thus, homology between many protein groups in MSP and snake venom main toxin proteins was observed. SVMP alignment with MSP metalloprotease indicated conservation in domains known in SVMP to be vital for toxicity: the metalloproteinase, disintegrin and cysteine-rich domains (Wagstaff et al. 2009). In addition, these domains have been previously identified as a part of the EoSVMP string, a multiepitope DNA immunogen, showing an intense and extensive immunoreactivity to SVMP (Wagstaff et al. 2006).

Similarly, serine proteases from MSP exhibited conservation with SVSP in domains that characterized by a catalytic mechanism that includes a highly reactive serine residue that plays a fundamental role in the formation of a transient acyl-enzyme complex. This complex is stabilized by the existence of histidine and aspartic acid residues within the active site (Serrano and Maroun 2005). C-type lectin demonstrated homology between MSP and snake venom c-type lectin in the CRD domain that plays an important role in pathology stimulated by this protein (binding to γ-carboxyglutamic acid (Gla)-containing domains of coagulation factors). These data represent a promising result for the identification of chimeric epitopes.

Immunoblotting results with snake antivenom (mono- and polyspecific) or toxin-specific IgGs supported the bioinformatics prediction of the presence of homology between these proteins that could be considered as experimental vaccine candidates in both organisms (Wagstaff et al. 2006; Harrison 2004). The cross-reactivity observed between MSP homogenate and antivenom/toxin-specific IgGs included the molecular weights of some of the proteins of interest in this study. Critically, these preliminary data suggest that epitopes can be identified and manipulated to produce chimeric immunising peptides representing both venom toxin and MSP.

An examination protein sequences from both MSP and venom reveals the cross-conserved expression of immunogenic domains that are naturally occurring B- and T-cell epitopes, as Figures 6.3–6.5 indicate. Using a bioinformatics approach, we constructed a chimeric epitope immunogen that may be able to generate antibody responses that neutralised

venom haemorrhagic toxins. NIBOVAC is a novel concept designed to exploit natural immunogens to stimulate sustained levels of protective immunity after initial vaccination against lethal toxin proteins. This, if successful, will overcome the decline of antibody concentrations after immunisation over time.

In this study, we designed chimeric epitopes that represented both MSP and snake venom toxins. These epitopes were of short, conserved peptide sequences predicted to be surface-exposed in toxin/MSP macromolecular structures, linked together to generate immunogens of 12aa length that can be conjugated to carbon nanotubes or linked to nanoparticles. All the selected epitopes were selected to stimulate Th1-associated immune responses preferentially, avoiding Th2-mediated hypersensitivity induction. In addition, all were selected with avoidance to human analogues. This was taken into account to avoid the development of an undesirable hypersensitivity reaction to natural mosquito bites and the induction of autoimmunity. These designed epitopes were sent to Dr Steven L. Cobb's lab at Durham University for epitope manufacturing and conjugation to carbon nanotubes. This vaccine delivery was selected because carbon nanotubes could serve as an excellent vehicle to administer vaccines (Pantarotto et al. 2003; Yang et al. 2007). Antigen conjugated to carbon nanotube vaccination may allow the effective utilisation of antigens that have previously been unable to induce adequate or appropriate responses, in addition to providing significant means of enhancing and modulating immune responses (Pantarotto et al. 2003).

Unfortunately, the time limitation of this PhD, as mentioned in the introduction section, restricted this study to designing the epitopes, as the end of the PhD period arrived before the delivery of the synthetic epitopes that had been conjugated to carbon nanotubes. Therefore, phase 2 of this study, which will validate the efficacy of NIBOVAC and test the protective efficacy of immune responses stimulated by mosquito saliva via challenging models of immunotherapy to mosquito bites, will be performed in a complementary study.

Overall, the work done at the Alistair Reid venom research laboratory has comprehensively defined the venom composition of medically important African Echis vipers and tested their immunological reactivity to venom toxin-specific antibodies using various approaches (Harrison 2004; Harrison et al. 2000; Wagstaff et al. 2006). In this study, I identified homologues of each pathogenic venom toxin group (metalloproteinases, phospholipase A_2s,

serine proteases and C-type lectins) in mosquito sialome datasets. It was determined that this cross-taxa sequence similarity extends to immunological cross-reactivity (immunoblotting) of mosquito salivary proteins (MSP) with snake antivenom and toxin-specific IgGs. I constructed *in silico* chimeric peptide immunogens from both organisms that showed antigenicity potential to stimulate immunity that, it is hoped, will be able to induce protective immunity by the completion of all phases of the study.

Chapter 7 General discussion

Epidemiological maps of neglected tropical diseases (NTDs) illustrated geographical overlap between the different diseases. The maps revealed that most of the diseases were concentrated in sub-Saharan Africa where the majority of the at-risk population, mortality, morbidity, and burden associated with the diseases reside. Furthermore, these populations are under great risk for snakebite that exert daily occupational hazard (Harrison et al. 2009). Emergent emphasis on integrated control of NTDs has created new awareness regarding the prospect and challenges of this approach (Utzinger et al. 2009). Co-endemicity of these diseases, as well as comorbidity, is of considerable public health relevance and offers opportunities for integrated control. Molyneaux et al. (2005) speculates that the administration of just four drugs may control seven NTDs in a cost-effective manner. These facts encouraged us to examine molecular parallels of the mechanisms used by a variety of tropical disease pathogens including parasites, snake venom toxins and hematophagous parasite vectors to access blood stream.

In accordance, biological studies of these diseases show that several of them may use similar mechanisms to complete their pathology. These mechanisms may include disturbing coagulation, disguising the immune system, or penetrating tissue compartments. Additionally, next-generation sequencing and integrated bioinformatic analyses have offered detailed and biologically relevant perceptions into the transcriptome of the different life stages of Schistosome, adult Fasciola, and venom composition. The bioinformatics analysis in this study illustrated that proteases utilized by venom and parasites belong to the same molecular function in terms of gene ontology (GO). In addition, the analysis also detected homology between proteases from venom and proteases from parasites.

Proteases play a major role in the pathology caused by parasites Schistosome and Fasciola, as well as venom. For example, parasite matrix metalloproteinases facilitates metalloprotease in the parasites' degradation of host collagen (Singh et al. 2006; Gomez et al. 1999). In the case of venom, metalloproteinases play an important role in inhibition of platelet aggregation and the degradation of blood clotting factors, which constitute systemic alterations, and thus cause systemic bleeding (Markland and Swenson 2013). Additionally, the metalloproteinases in venom also contribute to local tissue damage, probably by potentiating the hemorrhagic effect, which results from metalloproteinase in the local microvasculature and

degradation caused by matrix metalloproteinase (Gutiérrez and Rucavado 2000). Serine protease is also critical for both parasites and venom. Cercarial elastase, a serine proteinase secreted from the acetabular gland in the infective state of the parasite, is believed to initiate infection in schistosomes (Ingram et al. 2003; Ingram et al. 2012; Curwen et al. 2006). Other major mechanisms of initial infection include digestion, blood coagulation, immune response, and complement cascade. In the case of snake venom, serine proteases act on a variety of components of the coagulation cascade, the fibrinolytic and kallikrein–kinin systems, and cells, which triggers an imbalance of the prey's haemostatic system (Serrano and Maroun 2005; Mackessy 2009).

It is conceivable that transcriptomic representation of proteins in parasites or venom does not accurately represent true protein expression. A combination of transcriptomic and proteomic techniques is ideal. Therefore, we applied immunological cross-reactivity of parasites' proteins with antivenoms—known to have the ability to neutralize lethal toxins in snake venom—to measure the similarity between parasites and venom proteases based at the protein level. We demonstrated a presence of cross-reactivity toward parasite proteins in antivenoms. While immunological reaction did not prove to be specific to proteases from parasites, taking into account the fact that antivenoms are intended to attack the venom rather than only the toxins may clarify this discrepancy. Snake venom is a complex mixture with biologically active proteins, the vast majority consisting of peptides. Antivenoms, therefore, comprise antibodies to the majority of venom components, regardless of their toxicity or immunogenicity (Harrison 2004).

Interestingly, the analysis revealed that the majority of the identified parasites' proteins are important for the success of parasitism, or they were previously identified as vaccine or as potential therapy target. Of these, fructose 1,6 bisphosphate aldolase, and glutathione S-transferase proved to not only stimulate human immune responses against Schistosome, but can also mediate protection against a challenging infection, as well as decrease granuloma size in experimental schistosomiasis (Marques et al. 2008; Wright et al. 1991). Similarly, the analysis showed the identified enolase—a multi-functional protein localized on the cell surface and the tegument of helminthes—may bind to plasminogen, and may also have the ability to stimulate fibrinolytic activity for parasitic invasion and migration within the host (Liu et al. 2009). Manneck et al. (2012) demonstrated enolase as a potential drug target. They illustrated that the

antimalarial drug mefloquine has encouraging antischistosomal properties, killing haematophagous adult schistosomes and schistosomula by inhibiting enolase activity in the parasite (Manneck et al. 2012). In the same vein, studies identified Sm37-GAPDH as a vaccine candidate, showing mice and rats immunized with epitopes identified from Sm37-GAPDH were partially protected against a challenging infection (Argiro et al. 2000; Argiro and Doerig 2000). Other identified proteins from this study, such as Heat shock 70 (HSP70), calreticulin precursor, and 14-3-3 protein, are considered essential proteins in the pathology caused by Schistosome or Fasciola, and may be potential targets for therapy production (Schechtman et al. 2001; Kusel et al. 2007; Hedstrom et al. 1987; Ferreira et al. 2004).

Given the complexity of venom and comprehensive immune-binding nature of antivenom, the study revealed the identification of proteins rather than proteases cross-reacted with antivenom. To better define the specificity of the cross-reactive venom and parasite proteins we tested the ability of toxin-specific IgGs (raised against pathogenic venom proteins), to immunoreact with parasite proteases. Reactions of several IgGs raised against proteases from venom vary from parasite proteins. These IgGs represent proteases we are interested to compare between parasites and snake venom. The presence of immunological reaction in this experiment may provide insight into the effects of treating parasite stages with these toxin-specific IgGs. Toxin-specific IgGs proved to neutralize toxic effects of the specific toxin protease they raised against (Harrison et al. 2003; Harrison 2004; Wagstaff et al. 2006). If these IgGs were able to cross react with parallel proteases from parasites will they be able to prevent their effects in host? This may also set the foundations for testing the migratory or pathological ability of parasite stages, and perhaps for preparing affinity columns coupled with toxin-specific IgGs and identify bound proteins. Unfortunately, this was outside the scope of this study, as the sample size, presence of life stages of the parasites, and time limitation restricted this investigation.

The possibility of significant cross-reactivity of antivenom to parasite proteases, as illustrated in our experiments, led to investigating whether a similar immunoreactivity towards snake venom proteins exists in parasite-infected patients, and if so, to what extent the immunoreactivity can be sustained. The two parasites, Schistosome and Filaria, are characterized by a Th-2 type immune response in infected patients (Pearce and MacDonald 2002; Babu and Nutman 2012). This type of immune response is associated with elevated levels of IgE, high

production of IL-4, and eosinophilia (Paul and Zhu 2010; Riveau and Capron 2005; Babu and Nutman 2012). Both parasites are known to exert intense immunoregulatory effects on the immune system of the infected host. These include systemic immune-modulatory and immune-regulatory effects on the host system, against other infectious and non-infectious antigens (van Riet et al. 2007; Maizels and Yazdanbakhsh 2003; Wickelgren 2004). This disturbance in the immune system, caused by the presence of parasitic infection, represented a degree of difference between the immunoreactivity of human sera of infected and non-infected individuals. This observation may merit more relevance if we could investigate the immunoreactivity toward venom toxins in snakebite victims infected with parasitic diseases compared to those are not. It would be interesting to determine whether treatment with anti-parasitic drugs like ivermectin may exert down-regulatory effects on the patients' immune systems. It would also be relevant to determine if exposing individuals treated against parasitic diseases to snake venom might increase the risk of devastating effects (morbidity and mortality) resulted from snakebite on these populations. The result we demonstrated in Chapter 5 showed a complete loss of immunoreactivity toward the low molecular weight venom proteins, when probed with sera collected from treated filariasis patients and schistosomiasis patients living in villages with low prevalence of schistosomiasis.

The effects of the parasite infection on the immune system extend to the efficacy of therapeutic IgGs purified from infected patients. Studies demonstrated that IgGs purified from African patients infected with the malaria parasite exhibited a higher efficacy rate, compared to those purified from Americans or European donors for treatment purposes (Mekhaiel, Daniel-Ribeiro, et al. 2011; Rowe et al. 1968). We examined the reactivity of the IgGs toward antivenom, and although it showed immune cross-reactivity, it did not show a titre or robustness comparable with that from antivenom or toxin-specific IgGs neutralization.

In this study, we investigated possibilities of using homologous proteins in parasitic vector and snake venom as therapeutic applications. Molecules secreted and produced by these different pathogens exert complex effects on human physiological mechanisms, and thus may serve as the basis for novel therapeutic strategy. We constructed chimeric epitopes of homologous snake venom (*Echis ocellatus*) and mosquito salivary proteins as the primary vaccine. These designed chimeras when tested *in silico* exhibited promising antigenicity profiles.

This encouraging antigenicity, associated with that the domains from which these chimeric epitopes were designed, have been identified previously as potential vaccine candidates (Wagstaff et al. 2006; Harrison et al. 2003) (unpublished work done by Camila Renjifo in Alistair Reid venom research unit). If these epitope immunogens succeed in inducing and stimulating sustained levels of protective immunity against snakebites—when boosted with proteins delivered by mosquito bites—they will overcome the impracticality of conventional vaccination against snakebite. Vaccination against envenoming caused by snake venom is unfeasible, given the unlikelihood that a recalled immunological response could defend against the venom before the toxins' rapid lethal effects occurred (Müller et al. 2012). The presence of natural, seasonal boosting of immunological memory against venom toxins would hopefully protect a population at risk of snakebite against the lethal effects of venom. Ideally, this method would prevent, or at least reduce, the devastating pathology and death caused by snake venom at a low cost, with limited logistical complications.

Generally, this study demonstrated a presence of similarity between shared molecular molecules (proteases and other proteins) causing pathology in parasites, parasitic vectors and snake venom. These findings could become more relevant if field-based studies were to investigate the relation between parasitic infections and envenoming caused by snakebite. And test the effects of pre-existing parasite infections that cause minor bleeding in the infected host such as Schistosomes and hook worms may become a great deal worse subsequent to the haemorrhagic effects that follows systemic envenoming by some vipers – Echis. Other relevant experiments on living parasites include examining the effects resulting from treating these parasite stages with antivenom or toxin-specific IgGs on parasite migration and tissue destruction. Additional tests may consist of using secretory/excretory products of parasites to examine their similarity and cross-reactivity to antivenom and antitoxins, as well as using affinity purification to examine parasite proteins with binding ability to antitoxin IgGs. The area of NTD/snakebite biomedicine requires more research for the same factors demonstrated in the study, as pathogens causing NTDs affect the same population use molecules that may related to the same family or function in parallel way and they may cause pathologies that affect the same tissue or organ.

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APPENDICES

1.23 Appendix I: General stock solutions and buffers

1.24 Preparation of SDS-PAGE gel, electrophoresis and Western Blotting buffer

1. Resolving gel.

	15%		12.5%		10%	
	4 gels	**2 gels**	**4 gels**	**2 gels**	**4 gels**	**2 gels**
H_20	7.5ml	3.75ml	8.75ml	4.4ml	10ml	5ml
Tris pH 8.8	5ml	2.5ml	5ml	2.5ml	5ml	2.5ml
40% Bis Acrylamide	7.5ml	3.75ml	6.25ml	3.1ml	5ml	2.5ml
10% SDS	200µl	100 µl	200µl	100 µl	200µl	100 µl
10% APS*#	120µl	60 µl	120µl	60 µl	120µl	60 µl
TEMED*	14µl	7 µl	14µl	7 µl	14µl	7 µl

*APS and TEMED are activators so they need to be added last.

APS should prepare freshly each time 50 mg to 500 µl dH_2O.

2. Staking gel.

	2 gels	**4 gels**
H_20	2.5ml	5ml
Tris pH 6.8	1ml	2ml
40% Bis Acrylamide	350µl	700µl
10% APS	30µl	60µl
TEMED	5µl	10µl

3. 10x SDS running buffer.

Glycine	**720 g**
SDS	50 g
Tris-base	151 g
dH_2O	Up to 10 L

4. Coomassie blue stain.

Coomassie brilliant R 250	**4 mg**
Destain solution	2 L

5. Coomassie destain solution.

Methanol	**4.5 L**
Acetic acid	1 L
dH_2O	Up to 10 L

6. 10x PBS

Dissolve the following in 800ml distilled H_2O.

i. 80g of NaCl
ii. 2.0g of KCl
iii. 14.4g of Na_2HPO_4
iv. 2.4g of KH_2PO_4

Adjust pH to 7.4.

Adjust volume to 1L with additional distilled H_2O. Sterilize by autoclaving.

7. Native PAGE gel.

Resolving gel	2 gels	Stacking gel	2 gels
H_20	3.71ml	**H_20**	2.5ml
Tris pH 8.8	1.87ml	**Tris pH 6.8**	1ml
40% Bis Acrylamide	1.87ml	**40% Bis Acrylamide**	350µl
10% APS	37.5µl	**10% APS**	30µl
TEMED	3.75µl	**TEMED**	5µl

8. Native PAGE electrophoresis buffer.

Electrophoresis buffer	pH 8.3
dH_20	1.0 L
Tris base	3.0 g
Glycine	14.4 g

9. Transfer buffer.

Tris	2.03g
glycine	14.26g
H2O	800ml
Methanol	200ml

Preparation of sample homogenates

10. 2X protein loading buffer

dH_2O	**2.65 ml**
0.5 M Tris-HCl pH 6.8	1.25
Glycerol	2.5 ml
10 % SDS	3 ml
Saturated Bromo-blue solution (1mg in 1ml)	0.1 ml
5 % β-mercaptoethanol	0.5 ml

11. Non-reduced protein loading buffer

dH_2O	**Up to 20 ml**
0.5 M Tris-HCl pH 6.8	4 ml
Glycerol	5 ml
SDS	1.5 g
Saturated Bromo-blue solution (1mg in 1ml)	0.3 ml

12. Native protein loading buffer.

Sample buffer	
H_20	1.375 ml
Tris pH 6.8	0.625 ml
20% Glycerol	0.5 ml
0.02% Bromophenol Blue	40 µl of 5% Bromophenol Blue

13. *Fasciola hepatica* homogenate buffer.

20 mM $KHPO_4$ pH 7.4	**0.802 ml of 1M K_2HPO_4 + 0.198ml of 1M KH_2PO_4**
50 mM NaCl	0.5 ml of 1 M stock
10% glycerol	1ml
1% triton x 100	10 µl
1 mM DTT	100 µl of 100mM stock (154 mg in 10 ml dH_2O)
dH_2O	Up to 10 ml (~7.39 ml)

ELISA buffers

1. TBST buffer

Tris-HCl, pH 8.5	**10mM**
NaCl	150mM
Tween 20	1%

2. Citrate buffer

Citric acid	**525mg**
H2O	50ml

3.Coating buffer

Na2CO3	**1.59g**
NaHCO3	2.93g
NaN3	0.2g
H2O	1L

1.25 Appendix II: LC-MS/MS identification of cross-reacted parasites proteins

Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest	
									Ion score	Exp value	Probability	Xcorr
1	**35 - 50 kDa**	**Cercaria**	**Fructose-bisphosphate aldolase (EC 4.1.2.13)**	(P53442)	760.79	2	1	GILAADESTATMGKR	83	1.07432 E-05	61.59	4.91
					683.06	2	1	GILAADESTATMGK	73	1.21E-04	65.71	3.65
					635.13	2	2	FEGNMGTTLGDK	67	5.22E-04	21.91	3.39
					717.61	2	3	AYTPQENALATVR	66	5.64E-04	6.89	3.27
					514.56	2	4	IAQAICAPGK	53	1.35E-02	1.00	3.25
					691.20	2	5	ENVHAAQEELLK			10.13	3.19
					781.74	2	6	KAYTPQENALATVR				
1,4	**50 kDa**	**SEA**	**Actin-2**	(P53471)	977.87	2	1	VAPEEHPVLLTEAPLNPK	61	5.29E-04		
					1056.1	3	2	TTGIVLDSGDGVTHTVPIYEGY ALPHAILR			27.71	3.49
					977.87	2	1	VAPEEHPVLLTEAPLNPK			1.00	3.27
					566.94	2	3	GYSFTTTAER			54.16	3.06
					586.63	2	4	HQGVMVGMGQK			1.00	2.95
					599.73	2	5	AVFPSIVGRPR			34.33	2.72
					581.58	2	6	EITALAPSTMK			7.49	2.64
					489.01	2	7	AGFAGDDAPR			11.26	2.51

Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest	
									Ion score	**Exp value**	**Probability**	**Xcorr**
4	**50 kDa**	**Schistosomula**	**Enolase (EC 4.2.1.11) (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase)**	(Q27877)	530.54	2	1	IEEELGTAAK	62	2.18E-03		
					551.53	2	2	GVLTAVSNVNK			30.89	3.41
					595.11	2	3	AGAAEAGLPLYR			11.68	3.16
					530.54	2	1	IEEELGTAAK			7.53	2.95
					694.72	2	4	GNPTVEVDLKTSK			1.00	2.16
4	**35 kDa**	**Schistosomula**	**Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH) (Major larval surface antigen) (P-37)**	(P20287)	894.53	3	1	KVIISAPSADAPMFVVGVNENSYEK	77	2.27E-05		2.57
					724.08	2	2	GAMQNIIPASTGAAK	64	1.02E-03		2.55
					715.61	2	2	GAMQNIIPASTGAAK	63	1.03E-03		2.55

					889.19	3	1	KVIISAPSADAPMFVVGVNENSYEK	57	2.07E-03			2.55
Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest		
									Ion score	**Exp value**	**Probability**	**Xcorr**	
					911.35	3	3	VIHDKFEIVEGLMTTVHSFTATQK	55	3.84E-03			2.49
					1064.76	2	4	SMSVVSNASCTTNCLAPLAK	53	8.14E-03			2.44
					778.87	3	5	LTGMAFRVPTPDVSVVDLTCR	52	8.94E-03			2.40
					911.35	3	3	VIHDKFEIVEGLMTTVHSFTATQK			33.60		2.34
					894.53	3	1	KVIISAPSADAPMFVVGVNENSYEK			6.29		2.33
					778.87	3	5	LTGMAFRVPTPDVSVVDLTCR			62.50		2.29
					889.19	3	1	KVIISAPSADAPMFVVGVNENSYEK			11.56		2.28
					899.12	2	6	LVSWYDNEFGYSCR			75.40		2.24
					668.84	3	7	DSTHGTFPGEVSTENGKLK			27.95		2.23
					1269.46	2	1	VIISAPSADAPMFVVGVNENSYEK			24.46		2.17
					815.82	2	8	VVDLITHMHKVDHA			13.61		2.00
					640.72	3	7	RDSTHGTFPGEVSTENGK			1.00		1.86
					588.35	3	7	DSTHGTFPGEVSTENGK			1.12		1.84
					916.70	3	3	VIHDKFEIVEGLMTTVHSFTAT			1.86		1.80

								QK				
					808.29	2	8	VVDLITHMHKVDHA			30.85	1.74
					779.70	2	5	VPTPDVSVVDLTCR			53.40	1.32
					715.61	2	2	GAMQNIIPASTGAAK			17.51	
Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest	
									Ion score	**Exp value**	**Probability**	**Xcorr**
					724.08	2	2	GAMQNIIPASTGAAK			10.22	3.41
					539.15	3	8	VVDLITHMHKVDHA			18.11	3.16
					669.11	3	7	DSTHGTFPGEVSTENGKLK			5.84	2.95
					633.65	2	9	GASYEEIKAAVK			27.78	2.16
					598.13	2	9	LGKGASYEEIK			17.02	
					669.13	3	7	DSTHGTFPGEVSTENGKLK			1.00	
					589.08	2	10	AGISLNNNFVK			1.00	
					715.61	2	2	GAMQNIIPASTGAAK			1.00	
					591.96	3	2	DGRGAMQNIIPASTGAAK			1.00	
					715.11	2	2	GAMQNIIPASTGAAK			1.00	
					605.14	2	8	VVDLITHMHK			7.15	
					715.56	2	2	GAMQNIIPASTGAAK			1.56	
					879.79	2	2	DGRGAMQNIIPASTGAAK			1.00	
					715.69	2	2	GAMQNIIPASTGAAK			1.00	
					1265.51	1	9	GASYEEIKAAVK			1.00	

					715.62	2	2	GAMQNIIPASTGAAK			1.00	
					715.28	2	2	GAMQNIIPASTGAAK			1.00	
					1063.26	2	4	SMSVVSNASCTTNCLAPLAK			1.00	
Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest	
									Ion score	**Exp value**	**Probability**	**Xcorr**
					1429.59	1	2	GAMQNIIPASTGAAK			1.00	
					410.01	2	11	VGINGFGR			8.90	
					715.85	2	2	GAMQNIIPASTGAAK			1.00	
					510.09	2	11	AKVGINGFGR			2.12	
					589.56	2	10	AGISLNNNFVK			16.82	
					1430.56	1	2	GAMQNIIPASTGAAK			1.00	
					715.62	2	2	GAMQNIIPASTGAAK			1.00	
					812.29	1	12	VIPALNGK			1.00	
					1177.47	1	10	AGISLNNNFVK			1.00	
					1176.51	1	10	AGISLNNNFVK			1.00	
					811.32	1	12	VIPALNGK			1.00	
					812.29	1	12	VIPALNGK			1.00	
1,3	**50 kDa**	**Schistosoma adult**	**Calreticulin precursor (SM4**	(Q06814)	746.18	2	2	DNPEYKGEWTPR	55	7.22E-03		

			protein)									
					721.06	2	2	IMFGPDICGMATK			54.86	3.14
					642.05	2	2	SPVDPIEDLGLK			10.79	2.82
					746.18	2	2	DNPEYKGEWTPR			12.22	2.60
					726.24	1	1	FYGIAR			1.00	1.42
Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest	
									Ion score	**Exp value**	**Probability**	**Xcorr**
					890.20	1	1	TIPDMDAK			1.00	1.12
2	**15 kDa**	**Schistosoma adult**	**Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8) (PPIase) (Rotamase) (Cyclophilin) (Cyclosporin A-binding protein) (p17.7) (Smp17.7)**	(Q26565)	840.65	2	2	IIFELFNDVPDTTR	73	9.16E-05		
					1065.23	2	2	IIPGFMCQGGDFTNGDGTGGK	55	5.00E-03		
					1065.29	2	2	IIPGFMCQGGDFTNGDGTGGK	54	6.71E-03		
					840.645	2	2	IIFELFNDVPDTTR			49.40	3.83
					555.405	2	2	IIIEDCGEC			11.91	3.01

					635.060	2	2	AFFDIKAGDER			7.37	2.98
					1065.22	2	2	IIPGFMCQGGDFTNGDGTGGK			63.39	2.90

Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest	
									Ion score	**Exp value**	**Probability**	**Xcorr**
					713.170	2	2	NNFGYKGSVFHR			12.95	2.62
					840.155	2	2	IIFELFNDVPDTTR			36.01	2.61
					1065.29	2	2	IIPGFMCQGGDFTNGDGTGGK			11.75	2.41
					458.450	2	2	VVSGIDVVK			1.00	2.29
					916.365	1	1	VVSGIDVVK			1.00	2.01
					740.230	1	1	AFFDIK			1.00	1.86
					915.395	1	1	VVSGIDVVK			1.00	1.83
					1109.19	1	1	IIIEDCGEC			7.15	1.78
					1108.18	1	1	IIIEDCGEC			4.91	1.76
					740.26	1	1	AFFDIK			1.00	1.47

					0							
	59 kDa	**FAS**	**Heat shock 70 kDa homolog protein (HSP70) (Major surface antigen)**	(P08418)	585.560	2	1	DAGAIAGLNVLR	85	7.88837E-06		
					1040.26	2	2	VYQAGGMPGGMHEASGAGGGSGK	76	3.7231E-05		
					826.095	2	3	NQVAMNPTNTVFDAK	67	4.26E-04		
Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest	
									Ion score	**Exp value**	**Probability**	**Xcorr**
					846.745	2	4	STAGDTHLGGEDFDNR	64	7.93E-04		
					676.040	2	5	NSLESYVYTMK	63	1.18E-03		
					642.150	2	6	MKEVAESYLGR	58	3.76E-03		
					936.810	2	7	MDKSQIHDIVLVGGSTR	56	5.14E-03		
					585.560	2	1	DAGAIAGLNVLR			23.83	4.23
					642.150	2	6	MKEVAESYLGR			48.69	4.09

					676.040	2	5	NSLESYVYTMK			9.04	4.06
					846.745	2	4	STAGDTHLGGEDFDNR			53.19	3.85
					1032.23	2	2	VYQAGGMPGGMHEASGAGGGSGK			48.70	3.60
					624.815	3	7	MDKSQIHDIVLVGGSTR			1.00	3.40
					615.060	2	8	VEIIANDQGNR			1.00	3.32
					911.500	2	3	NQVAMNPTNTVFDAKR			33.53	2.94
					737.085	2	9	TTPSYVAFTDSER			3.67	2.82
					692.760	2	10	IPESDRQVIISK			12.87	2.74
					563.145	2	11	ICVEYKGEK			10.67	2.52
					992.205	2	12	TVSDAVITVPAYFNDSQR			6.89	2.50
					720.655	2	13	RFDDPSVQSDMK			5.04	2.09
					860.120	1	14	GPTIEEVD			1.00	1.45
Antivenom	Band	Organism	Protein	Accession no.	m/z (Da)	Z (+)	Peptide number	MS/MS derived sequence	Mascot		Sequest	
									Ion score	**Exp value**	**Probability**	**Xcorr**
					858.230	1	15	GTLDPVEK			1.00	1.43

1	**25 kDa**	**Schistosoma adult**	**14-3-3 protein homolog 1**	(Q26540)	612.555	2	2	YLAEVATDDAR	95	7.40E-07		
					797.220	2	2	ATTAAENLPTTHPIR	65	6.72E-04		
					636.775	3	3	YLAEVATDDARTEVVQK	43	9.78E-02		
					612.555	2	2	YLAEVATDDAR			21.07	3.71
					797.220	2	2	ATTAAENLPTTHPIR			54.58	3.32
					636.775	3	3	YLAEVATDDARTEVVQK			17.62	3.06

Table 0.1 LC-MS/MS identification of cross-reacted parasites proteins: Proteins with immunological cross-reactivity with snake venom were excised and trypsin-digested from individual parasite samples. The table includes; a list of peptide sequences derived by mass spectrometry for each protein of interest, numbered to link it to the alignment in the following section, and the accession number of the protein, which corresponds to the closest protein match identified by BLAST search. The numbers in the first column refer to the antivenom the corresponding protein reacted with: 1) EchiTAb G, 2) EchiTAb-Plus-ICP, 3) SAIMR polyvalent, 4) CSL polyspecific. Ion score and E value indicate protein identified by Mascot algorithm, and Probability and XCorr correspond to protein identification by Sequest algorithm.